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CVII. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

III. A METHOD FOR THE MEASUREMENT OF RESPIRATORY QUOTIENT IN SERUM.

By FRANK DICKENS[1] and FRANTIŠEK ŠIMER.

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In Part I of this series [Dickens and Šimer, 1930] the principles applicable to the measurement of the respiratory quotient of surviving tissues *in vitro* were summarised. It was concluded that the only existing principle by which the true R.Q. could be measured correctly, regardless of changes in acid-base equilibrium, was that in which the tissue is suspended in a CO_2-free medium, such as a Ringer solution buffered with phosphate, the gas-space containing pure CO_2-free oxygen. A reliable method involving such a principle was there described.

The method gives accurate results and is convenient and simple to use. Nevertheless, there are two obvious objections which may be brought against the conditions of the tissue in such a method; firstly, the low CO_2 tension, which approaches zero in the medium, and secondly, the fact that the whole of the buffering is due to phosphate, whilst in the body phosphate buffers are, at least quantitatively, much less important. Above all, the phosphate method cannot be applied if serum or other similar body-fluids are used as medium. There can be no doubt that at any rate in the case of certain delicate material, such as germinal and embryonic tissues, and probably for other tissues also, serum is a more suitable medium than a simple salt solution.

When an attempt is made to find a method which might be applied to the measurement of respiration and respiratory quotient in such a medium, very great practical difficulties arise. These are due to the circumstance remarked on by Warburg [1925] who wrote: "Up to now, no method exists for the estimation of the respiration CO_2, when fluids are employed whose bicarbonate content corresponds to that of serum, *i.e.* under conditions which are physiological for cells of the higher animals."

In the present paper, a method is described whereby the CO_2 formed in respiration by a piece of tissue suspended in such a medium may be directly measured, independently of any changes whatever in the bound CO_2, whether these changes arise from production or consumption of acids by the tissue, or

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whether they are due to retention of CO_2 by the buffer substances contained in the medium. The oxygen consumption of the same piece of tissue is given simultaneously by manometric readings, and hence the R.Q. is accurately determined. The method may therefore be used for determining the R.Q. and respiration, either with simple Ringer solution containing bicarbonate, or with serum or other similar fluid in equilibrium with the physiological tension of CO_2. A great advantage of the method is that exactly the same technique and calculation may be used with equal accuracy either for Ringer solution or serum, and no corrections, empirical or otherwise, are needed for the "retention" in the latter medium.

Principle. The method is a manometric one. A thin slice of the tissue is suspended in serum or Ringer solution in a vessel of special design, filled with a mixture of oxygen with the required percentage of CO_2. The vessel and contents, connected with a simple Barcroft-Warburg manometer, are shaken in a thermostat as in Warburg's method [1925]. If we denote the total pressure-change read on the scale of the manometer during the experimental period by h, this pressure, which may be either positive or negative, is made up of the following:

(1) h_{O_2} = pressure-change caused by disappearance of oxygen consumed in respiration;

and (2) h_{CO_2} = pressure-change due to respiratory CO_2;

and (3) h_b = pressure-change due to alteration in the amount of combined CO_2 during the experiment.

These magnitudes are related to the observed pressure-change h, by the following equation:

$$h = h_{O_2} + h_{CO_2} + h_b \qquad \text{.....(1).}$$

In this equation all pressures must be algebraically reckoned, h_{O_2} being always negative, h_{CO_2} positive, and h_b either positive or negative according to whether the total amount of chemically combined CO_2 is diminished or increased.

The magnitude of h_b is determined by liberation of the whole of the bound CO_2 present at the beginning and end of the experiment by acidification with excess of strong acid.

In order to determine h_{CO_2} a new principle has been introduced. A direct determination is made of the change during the experimental period in the total amount of CO_2, bound and free, present in the vessel. This quantity is the amount of CO_2 respired during the experiment and is denoted by x_{CO_2}. It is important to emphasise that by the determination of this magnitude, three of the four quantities in equation (1) are known, namely, h, h_{CO_2} and h_b. Hence the value of h_{O_2} is determined, and therefore the amount of respired oxygen (x_{O_2}) is also known.

Both x_{O_2} and x_{CO_2} being known, the R.Q. $= -x_{CO_2}/x_{O_2}$.

It will be noted that the above principle is quite general for any medium.

Method.

Apparatus and solutions. The special vessels[1] (Fig. 1) used to contain the tissue and solutions are attached by means of the ground-joint to Barcroft manometers. The vessels are conical in shape, with as nearly as possible equal volumes of about 20 cc. They contain an inner separate conical part (*A*) which receives the piece of tissue and the medium (1·5 cc. serum or Ringer solution).

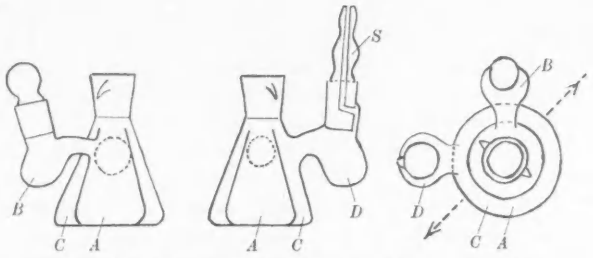

Fig. 1. The dotted line in the plan shows direction of shaking.

Communicating with compartment *A* by a fused-through join is the bulb *B*, containing excess of acid (0·1 cc. 3*N* HCl) which may at any desired moment be tipped into *A* by inclining the vessel. In this way, the respiration is stopped and the total bound CO_2 is liberated.

The outer annular portion *C*, and the bulb *D* communicating with it, serve for the absorption of the whole of the CO_2 contained in the vessel. For this purpose, use has been made of a principle of gas-analysis described by Krebs [1930]. The part *C* contains 2 cc. of a solution of $KMnO_4$ (*M*/5 with *M*/500 H_2SO_4), whilst bulb *D* has 0·2 cc. 30 % NaI, the latter solution being acidified to *N*/500 with H_2SO_4 immediately before use. When the contents of the bulb *D* are tipped into the part *C*, the iodide reacts with the $KMnO_4$ and a strongly alkaline solution results. The free CO_2 is thus rapidly and quantitatively absorbed.

The stopper *S* is of the type used by Warburg and Kubowitz [1929] for the determination of oxygen in gas-mixtures. It is closed by turning the stopper under the water of the bath, and permits the streaming of gas through the vessel whilst the latter is being shaken in the thermostat. Its use is essential for the accuracy of the method. An ordinary glass stopper is used for the other bulb (*B*).

Manometric fluid. The usual Brodie fluid (sp. gr. 1·033) is unsuitable for these experiments since its use would necessitate inconveniently long manometers in order to measure the large pressure changes involved. According to the carbon dioxide tension in the vessel, one or other of two different fluids has been found satisfactory.

[1] The vessels were made for us by Messrs C. Dixon and Co., 27, Devonshire Street, London, W.C. 1.

(1) For CO_2 tensions of the order of that occurring in serum, Clerici solution as used by mineralogists (a mixture of thallium malonate and thallium formate of sp. gr. approximately 4) is used. Sodium tauroglycocholate (0·1 %) is dissolved in the solution before use and, after filtration, the sp. gr. of the mixture at the prevailing temperature of the laboratory is accurately determined.

(2) In some experiments where a lower CO_2 tension than the physiological is employed, a solution of lead perchlorate, sp. gr. 2·07 [Krebs, 1930], has been used instead of the above solution.

These solutions must be protected from accidental dilution by droplets of water by means of loosely-fitting glass caps on the open ends of the manometers and by cotton-wool plugs inserted loosely in the tubes supplying the moist gas stream to the vessels. If new rubber tubing is used for the reservoirs of the manometers, it should be artificially aged by soaking in Clerici solution before use to remove traces of an impurity which otherwise slowly reacts with the solution, forming a dark-coloured deposit on the glass.

Supply of gas mixture. The gas mixture stored under pressure in the usual type of commercial gas-cylinder is first passed through a simple **T**-tube safety device, consisting of a vertical tube immersed to a suitable depth in water, which controls the maximum pressure of gas in the manometers. It then passes through a little water contained in a bubbler immersed in the bath, and the moist gas is distributed to the manometers through a six-way tube. Before entering the manometers the gas passes through the small tubes containing the cotton-wool plugs already mentioned; these tubes are attached by flexible rubber connections to the gas supply in order to allow vigorous shaking during the passage of the gas.

Procedure. For each determination, two vessels with tissue are necessary, or more if duplicate estimations are to be made. The solutions are first pipetted into the vessels as described above, serum having first been approximately saturated with the gas-mixture used in the experiment. Special care is necessary to measure exactly equal volumes of the serum or $NaHCO_3$-Ringer into all vessels. The fresh tissue is then cut with the razor in the usual way, with care to cause minimal damage to the tissue and to secure slices sufficiently thin to permit complete diffusion of metabolites [Warburg, 1925]. Before being transferred to the vessels, the slices are first suspended in serum or $NaHCO_3$-Ringer solution and a stream of 5 % carbon dioxide is passed briskly through for some 5 minutes, in order to arrive at a uniform preliminary condition in the various slices. They are then weighed in the moist state on a torsion balance, the weight for each vessel being equal, and the amount sufficient to give a respiration of some 100 $mm.^3$ per hour. After weighing each piece of tissue it is quickly rinsed in a $NaHCO_3$-free salt solution. This stage is not essential, but tends to lead to more consistent figures for the $NaHCO_3$-introduced with the tissue into the vessel. The tissue is then placed in the central compartment of the vessels, into the serum or $NaHCO_3$-Ringer. The vessels are attached to

their respective manometers and are together put into the bath with their stoppers closed. A third vessel without tissue and containing only the $KMnO_4$ and NaI is used for the analysis of the gas mixtures, and as usual the bath also contains a thermobarometric control vessel. The gas stream (see above) is now connected to the upper tubes of all manometers and a positive pressure of gas is set up in the vessels. The stoppers S of the vessels are now opened under the water, without removing the vessels from the bath, until a vigorous gas-stream is seen to bubble through the water of the bath. At the same time it is necessary to make sure that the vessels are firmly fixed to their manometers by means of their ground-joints. The shaking apparatus is now set in motion and the time noted. A period of 10 minutes, during which a steady stream of gas passes and the shaking is maintained at the rate of 90 swings per minute, suffices for thermal and gaseous equilibrium to be established. This rate of shaking must be used throughout the experiment. During the preliminary period the levels of the manometric fluid are roughly adjusted to a suitable height. The gas-stream is now stopped by turning the stoppers S without removing the vessels from the bath, the shaking being momentarily interrupted for this purpose. The upper taps of the manometers are closed, the tubing used for the gas-supply disconnected, and the final adjustment of level in the manometers is made. Shaking is recommenced, and the first reading of all vessels is taken 15 minutes from the time of first putting into the bath. Immediately after taking this reading, the acid from bulb B is at once tipped into the control vessels, which are momentarily removed from the bath for this purpose. The positive pressure caused by this acidification is read off and is denoted by h_{B_0} in the Protocol at the end of this paper, in which vessels Nos. 7 and 11 are the controls on the preformed CO_2, and Nos. 9 and 10 are the duplicate experimental vessels used for a double determination of R.Q., whilst No. 12 is solely for analysis of the gas-mixture from the cylinder.

During the experimental period of about 3 hours, the shaking of manometers is interrupted at intervals of half hour in order to follow the change of pressure in Nos. 9 and 10, the difference between the initial pressure and final pressures at the end of the 3 hour period, corrected of course for any small changes shown by the thermobarometer vessel, gives the value of h. Immediately after reading this quantity, the acid is tipped into the experimental vessels, causing a positive pressure (h_{B_1}) which is read off. After adding the acid, about 10 minutes is sufficient for the complete evolution of CO_2.

The whole of the CO_2 in the vessels is now in the free state, and is determined in all vessels, including No. 12, used for analysis of the gas-mixture, by tipping the iodide into the $KMnO_4$.

The absorption of CO_2 is complete in some 20 minutes, and the resulting negative pressures, H_0, H_1, and H_{gas} are then read off on the respective manometers of the control, experimental, and gas-analysis vessels.

Calculation. The basis of the calculation is the relationship given in equation (1):

$$h = h_{O_2} + h_{CO_2} + h_b \qquad \text{.....(1).}$$

Since the volumes of gas corresponding to these heights are merely the products of these heights in mm. and the appropriate vessel-constants, equation (1) may be re-written in the form:

$$h = \frac{x_{O_2}}{k_{O_2}} + \frac{x_{CO_2} + x_b}{k_{CO_2}} \qquad \text{.....(2).}$$

Vessel constants. For a manometric fluid of specific gravity S, the calculation of the vessel-constants for oxygen and carbon dioxide is as follows:

$$k = \frac{V_G \times (273/T) + V_F . \alpha}{760 \times (13{\cdot}60/S)},$$

where $V_G + V_F$ = total volume in mm.^{3} of vessel and capillary of manometer to the zero mark, V_F = volume of liquid placed in manometer vessel and α = Bunsen solubility coefficient of oxygen or CO_2 respectively. S must be accurately determined for each batch of manometer fluid prepared.

In the following calculations, quantities determined in the experimental vessel I (at time t_1), and at time t_0 in the control (vessel O) are denoted by the suffixes 1 and 0 and the vessel-constants for carbon dioxide by k_1 and k_0 respectively, whilst k_{O_2} is the constant for oxygen relating to the experimental vessel.

Determination of x_{CO_2}. The total amount of CO_2 in the vessel is made up of the following: (1) CO_2 introduced as gas at the time of filling the vessel, (2) bound CO_2 introduced with the tissue and serum or Ringer bicarbonate, (3) CO_2 formed in respiration. Each of these will now be considered.

(1) Since all the vessels are filled with the same gas-mixture under the same pressure, all contain the same percentage of CO_2 at the time of closing the taps. By absorption of this amount of CO_2 the negative pressure H_{gas} is set up, which is related to the percentage of CO_2 in the gas-mixture used by the following equation [Krebs, 1930]:

$$\% \ CO_2 = \frac{H_{gas} \times 100}{P_o - p_w} \qquad \text{.....(3),}$$

where P_o = total pressure inside the vessel at the beginning of the experiment, and p_w = pressure of saturated vapour above the solutions in the vessel, all pressures being reckoned as usual in heights of manometer fluid.

This equation shows that H_{gas} is independent of the size of vessel or amount of fluid contained in it, and for a certain gas-mixture is constant if the total pressure in the vessels is the same. In any particular experiment this is easily realised by making the initial levels of manometer fluid in the two limbs approximately equal. The actual amount of CO_2 is of course dependent on the size of the vessel and amount of fluid, and is given by $k_{CO_2} . H_{gas}$.

(2) If the quantities of solution and tissue are the same in experimental and control vessels, the amount of bicarbonate introduced is the same in both vessels and is affected to the same extent in both vessels up to the time t_0.

(3) The same applies to respiration, which up to the time t_0 is the same in both vessels. In any case, this term is very small, since it concerns only the amount of respiratory CO_2 produced in the interval between stopping the gas-stream and t_0, which is in general only 5 minutes or less.

The volume of CO_2 produced between the times t_0 and t_1 in the experimental vessel, *i.e.* respiratory $CO_2 = x_{CO_2}$, is given by the difference in initial and final contents of total CO_2 in the vessel. If the vessels were of exactly the same size, x_{CO_2} would be simply the difference in the amounts of CO_2 present in the control vessel at t_0 and the experimental vessel at t_1. Since in practice we are dealing with vessels of slightly different volume, this must be considered in the calculation, which is carried out by either of the two principles described below.

First principle.

The quantities actually measured are H_1 and H_0, from which we may at once subtract H_{gas}, since this is the same in both vessels. Hence, apart from the amount of free CO_2 introduced in the gas-mixture, the amounts of CO_2 at time t_0 and t_1 are:

$$(H_0 - H_{gas})\, k_0 \text{ and } (H_1 - H_{gas})\, k_1$$

respectively. The difference between these two amounts is the respiratory CO_2 between time t_0 and t_1, hence:

$$x_{CO_2} = (H_1 - H_{gas})\, k_1 - (H_0 - H_{gas})\, k_0 \qquad \text{.....(4).}$$

Determination of x_{O_2}. From equation (2):

$$x_{O_2} = \left(h - \frac{x_{CO_2} + x_b}{k_1}\right) k_{O_2} \qquad \text{.....(5).}$$

In this equation, x_b is the change in bound CO_2 during the experimental time $t_1 - t_0$. If B_0 = initial amount of bound CO_2 at the time t_0, and B_1 = bound CO_2 at time t_1 in the experimental vessel, then:

$$x_b = B_0 - B_1 = h_{B_0} k_0 - h_{B_1} k_1 \qquad \text{.....(6).}$$

The values x_{CO_2} and x_b and h in equation (5) being known, x_{O_2} is thus determined, and R.Q. $= -x_{CO_2}/x_{O_2}$ can at once be calculated.

Second principle.

By substituting the observed pressure changes from equations (4) and (6) in equation (5), this may also be written in the form:

$$h_{O_2} = h - H_1 + h_{B_1} + H_{gas}\{1 - (k_0/k_1)\} + (H_0 - h_{B_0})(k_0/k_1) \quad \text{...(7).}$$

Hence if $k_0 = k_1$, $h_{CO_2} = H_1 - H_0$ (from equation (4)) .....(4*a*),

and $h_{O_2} = h - H_1 + H_0 + h_{B_1} - h_{B_0}$ (from equation (7)) .....(8).

If the volumes of the two vessels are nearly the same, this condition can be achieved in practice, by varying the volume of liquid in these two vessels so that k_{CO_2} is the same for both. This modification makes the separate determination of H_{gas} unnecessary, provided as before that the quantities of tissue and serum taken are equal in the two vessels.

Notes.

(1) *Time of saturation with gas-mixture.* The time allowed is sufficient to attain complete filling of the vessel with the gas-mixture, and in control experiments the analysis was unchanged by prolonging the passage of gas for a greater time.

(2) *Control experiments with $NaHCO_3$ solutions.* In the absence of tissue in the vessel, x_{CO_2} is zero and $H - H_{gas} = h_B$, where h_B is the pressure change caused by the liberation of CO_2 from the bicarbonate taken. In numerous control experiments h_B and B $(= h_B.k_{CO_2})$ have been determined (*a*) by acidification and (*b*) from measurements of H and H_{gas} with an accuracy governed only by the accuracy of the manometer readings, *i.e.* $\pm$ 0·5 mm. manometer fluid.

(3) *Blank correction for tipping of NaI.* It should be mentioned that in determining the actual percentage of CO_2 in the vessel, it is necessary to apply the small correction of a few mm. mentioned by Krebs, due to the slight change of pressure which accompanies the mixing of the NaI and $KMnO_4$ and having no relation to the absorption of CO_2. In the method described, where the absolute percentage of CO_2 is not required, this correction is unnecessary, since it cancels out, as is clear from equations (4) and (8).

(4) *Magnitude of respiration and gaseous diffusion.* The fundamental principle underlying manometric measurements is the existence of a virtually complete equilibrium between the liquid and gaseous phases. The quantities of tissue and rate of shaking referred to above are suitably chosen to ensure this, and should be adhered to.

(5) *Correction for different weights of tissue.* With a little practice it is usually possible to weigh sufficiently nearly equal pieces of wet tissue into each vessel to render special correction for this point unnecessary; particularly as the magnitude of the correction for tissue taken from the control vessel is always small compared with the actual measurement in the experimental vessel. In all cases, however, the dry weight is accurately determined by drying to constant weight at 110°, since it is required for calculation of Q values [Warburg, 1930] and also as a check on the equality of the wet weights taken.

Should there be an appreciable difference between the dry weights of tissue in the control and experimental vessels, the correction may be applied in the following way. In the first place, the total bicarbonate content of the volume of original medium used is determined in the usual way. Denoting

this by B_M mm.^{3} and the weights of tissue by m_1 (experimental vessel) and m_0 (control vessel) then

$$x_b = B_M + (B_0 - B_M)\, m_1/m_0 - B_1,$$
$$x_{CO_2} = (H_1 - H_{gas})\, k_1 - B_M - (H_0 k_0 - H_{gas} k_0 - B_M)\, m_1/m_0.$$

The remainder of the calculation is as given above.

Notes on the determination of glycolysis or oxidative removal of lactic acid.

(1) *In bicarbonate-Ringer solution.* In solutions containing no other buffer besides bicarbonate, the change in bound CO_2, x_b, is exactly equal to the amount of acid formed or consumed by the tissue (x_G):

$$x_b = x_G.$$

x_G is positive if acid is formed, *i.e.* CO_2 liberated, and negative if acid is consumed (*i.e.* CO_2 bound). Both quantities are expressed in mm.^{3} according to Warburg [1930].

From x_G the value of aerobic glycolysis is calculated in the usual way:

$$Q_M^{O_2} = x_G/(m.t),$$

where m is the dry weight of the tissue in mg. and t the duration of the experiment in hours.

The method described may be applied with equal accuracy to the measurement of oxidative removal of lactic, pyruvic or other organic acids metabolised by tissues.

(2) *In serum.* In serum the decrease of the amount of bicarbonate, x_b, is smaller than the amount of fixed acid, x_G, formed by the tissue. The difference, $x_G - x_b = \Delta x$, is due to the presence of buffers other than bicarbonate, which (1) neutralise part of the acid formed in glycolysis, and (2) combine with a certain amount of CO_2 formed in respiration.

Δx must be determined experimentally. Two vessels, O and I, of the type described in this paper (see Fig. 1) are used for the determination. Vessel I is the same vessel as that used in the main experiment.

Exactly equal amounts of a solution of tartaric acid are pipetted into bulb B of both vessels and evaporated to dryness at 100° [Warburg, 1925]. The concentration of the acid and the amounts pipetted must be chosen so that the quantity in bulb B of both vessels is as nearly as possible equal to x_G ($> x_b$). Into bulb D of both vessels is now pipetted a solution of tartaric acid of such concentration and volume that the quantity of acid is exactly equal to the respiratory CO_2, x_{CO_2}, of the main experiment. This condition is easily fulfilled as x_{CO_2} is accurately known from the main experiment. The volumes of tartaric acid solution in B and D are checked by weighing the vessel before and after pipetting, and a correction is applied if necessary.

The central compartment (A, Fig. 1) contains 1·5 cc. of serum in vessel I and 1·5 cc. bicarbonate-Ringer in vessel O. Into the outer part C of both vessels bicarbonate-Ringer solution is pipetted, the volume measured being

such that the total volume of fluid V_F is in both equal to that in the main experiment (3·80 cc.).

Both vessels are now filled with the same gas mixture in the same way as in the main experiment. After equilibrium has been established a reading of pressure is taken and in both vessels the acid in D is tipped into C, and the dried acid in B is washed into A simultaneously. Denoting the total resulting pressures set up in vessels O and I by h_0 and Δp respectively, the amounts of CO_2 liberated are $h_0 k_0$ and $\Delta p . k_1$. The difference $h_0 k_0 - \Delta p . k_1 = \Delta x'$, is the amount of acid retained by the serum in vessel I under the conditions of this particular experiment.

This amount $\Delta x'$ is equal to Δx only if the quantity of acid in bulb B is exactly equal to x_G, *i.e.* if

$$\Delta p = \frac{x_{CO_2} + x_b}{k_1} \qquad \text{.....(9).}$$

In choosing the amounts of acid some preliminary experiments with varying amounts of acid in B may be necessary. The amount of acid in D is of course in these estimations kept constant and equal to x_{CO_2} in the main experiment.

If the amount of acid taken is not exactly the same as that in the main experiment, but the conditions approximate fairly closely to those prescribed by equation (9), the observed value of $\Delta x'$ may be corrected for the conditions of the main experiment as follows:

$$\Delta x = \frac{\Delta x'}{\Delta p} \cdot \frac{x_{CO_2} + x_b}{k_1} \qquad \text{.....(10).}$$

The use of the above principle of calculation of aerobic glycolysis in serum is made possible by the direct determination of x_{CO_2} by the new method, and has the following advantages.

(1) It is not necessary to calculate the retention for CO_2 from an observation of the retention for lactic acid, or *vice versa*.

(2) When both glycolysis and respiration occur together, as for example in tumour tissue, the effect of the lactic acid retention is to cause a change in the retention for CO_2 and *vice versa*, during the experiment. This mutual influence of the two retentions is allowed for in the above direct method of determination.

Accuracy. The probable error to be expected in the value of R.Q. may be conveniently considered on the basis of equations (4 *a*) and (8). Under these simplified conditions (weight of tissue, and constants for carbon dioxide equal in the different vessels) the relationship between the pressures directly observed and the R.Q. is as follows:

$$\text{R.Q.} = \frac{(H_1 - H_0)}{(H_1 - H_0) + h_{B_0} - h_{B_1} - h} \cdot \frac{k_{CO_2}}{k_{O_2}} \qquad \text{.....(11).}$$

In this equation the largest term, $H_1 - H_0$, is present in both numerator and denominator. This is very favourable to the accuracy of the method since any slight error in $H_1 - H_0$, due either to the error in readings themselves

($\pm$ 0·5 mm.), or to slight inequalities in the initial amount of CO_2 in the experimental and control vessels has a negligible effect on the value of R.Q.

The limiting factor in the accuracy is therefore the measurement of h_{B_0} and $(h_{B_1} + h)$. The difference of these two terms which occurs in the denominator of equation (11), is always smaller than $(H_1 - H_0)$, and the accuracy of measuring this quantity is controlled by

(1) accuracy of reading ($= \pm$ 0·5 mm.);

(2) accuracy in pipetting equal amounts of $NaHCO_3$ in the control and experimental vessels;

(3) agreement in bicarbonate content of the two pieces of tissue in the two vessels.

On the basis of the above error in scale-readings, and the experimentally observed accuracy in determining (2) and (3), and assuming a respiration of the order mentioned in this paper, the probable accuracy may be calculated to be 2 to 3 %, or 0·02 in the determination of R.Q. This order of agreement between duplicate determinations of R.Q. on the same tissue has been obtained in a number of experiments, which will form the subject of Part IV of this series.

We wish to express our indebtedness to Prof. O. Warburg, with whom we had the privilege of discussing the problem in the early stages of the development of the method.

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Protocol.

Rat liver in inactivated horse-serum containing 0·2 % glucose. Rat fasted 24 hours. Duration of experiment, 3 hours. Gas-mixture, oxygen with 4·50 % CO_2. Bicarbonate content of serum = 461 mm.³ per cc. Hence $p_H = 7{\cdot}41$. Temperature 38°.

Vessel No.	7	9	10	11	12
Total volume (cc.)	19·73	21·38	21·00	20·16	—
Solutions:					
A (Fig. 1)		1·5 cc. serum			—
B ,,		0·1 cc. HCl			—
C ,,		2·0 cc. $KMnO_4$			—
D ,,		0·2 cc. NaI			—
Weight of tissue:					
Wet (mg.)	80	90	90	90	—
Dry (mg.)	15	18·7	21·2	21·3	—

Gas passed through vessels in bath for 10 mins. Taps then closed and first reading taken after further 5 mins. (t_0).

Readings:

Pressure changes corrected for thermobarometer (mm. Clerici solution of sp. gr. 3·975).

	At t_0 tip B into A	—	—	Tip B into A	—
	$h_{B_1} = +113$	—	—	$h_{B_1} = +111$	—
	At t_1	$h = -30{\cdot}5$	$h = -34$	—	—
		Tip B into A	—	—	—
		$h_{B_1} = +103{\cdot}5$	$h_{B_1} = +105{\cdot}5$	—	—
		Tip D into C (all vessels)			
	$H_0 = 235$	$H_1 = 280$	$H_1 = 286{\cdot}5$	$H_0 = 234{\cdot}5$	$H_{gas} = 111$
Calculation:					
Vessel constants					
k_{CO_2}	6·17	6·72	6·60	6·31	—
k_{O_2}	—	5·92	5·84	—	—
k_{O_2}/k_{CO_2}	—	0·889	0·884	—	—
$h_b k_{CO_2} = B$ (mm.³)	697	696	696	700	—
$B_0 - B_1 = x_b$ (mm.³)	—	3	3	—	—
$(H - H_{gas})\, k_{CO_2} = C$ (mm.³)	764	1136	1157	779	—
$C_1 - C_0 = x_{CO_2}$ (mm.³)	—	**364**	**385**	—	—
$hk_{O_2} - k_{O_2}/k_{CO_2}(x_{CO_2} + x_b)$ $= x_{O_2}$ (mm.³)	—	**−508**	**−540**	—	—
Respiratory quotient:	—	**0·72**	**0·71**	—	—
Q_{O_2} (mm.³ oxygen respired per mg. tissue per hr.)	—	**−9·1**	**−8·5**	—	—

CVIII. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

IV. THE RESPIRATORY QUOTIENT IN BICARBONATE-MEDIA.

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The experiments described in Part II of this series [Dickens and Šimer, 1930] demonstrated the existence of a definite relationship between the aerobic and anaerobic metabolism of carbohydrate by normal tissues. As has often been pointed out, the figures for total oxygen consumed in respiration and those for anaerobic glycolysis appear to bear no mutual relationship whatsoever [*e.g.* Meyerhof, 1930, p. 178]. When, however, the previous observations were completed by the addition of measurements of the R.Q. and, instead of considering the relationship of the total respiration to the glycolysis, we compared the glycolysis with that fraction of the respiration corresponding to the oxidation of carbohydrate, it was then found that these two sets of values ran parallel in all the types of normal tissue studied. In tumours, on the other hand, there is a definite damage to the oxidation of carbohydrate, so that the amount of carbohydrate oxidised to CO_2 and water is smaller than would correspond with the high value of anaerobic glycolysis in such tissues.

In the above experiments, the tissue was suspended for the purpose of the measurements in a Ringer solution buffered with phosphate. It was considered desirable to ascertain if this relationship is generally applicable, and particularly whether it holds in the case when bicarbonate-media such as serum or bicarbonate-Ringer, are used. For this purpose it was necessary to evolve a method suitable for the measurement of R.Q. under these conditions. The present paper concerns the application of this method to the above problem and the comparison of the R.Q. and respiration of tissues in bicarbonate- and phosphate-media.

It may be stated at once that the conclusions of Part II hold good under the new conditions and are therefore independent of the particular medium used for the experiments. The detailed discussion of the results is left until after the description of the experiments.

Experimental.

The method used for the determination of R.Q., respiration and aerobic glycolysis has been fully described in Part III [Dickens and Šimer, 1931]. The tissue, cut into thin slices (except when, as with the retina or embryo, the intact material is suitable for use without cutting), is suspended in bicarbonate-Ringer solution [Warburg, 1930] or horse-serum, the latter being previously inactivated by heating to 56° for 1 hour. In all experiments glucose (0·2 %) was added to the medium before use. The serum used contained a normal amount of lactic acid (25–30 mg./100 cc.).

It is necessary to repeat here that, for the sake of accuracy in the R.Q., the total oxygen consumption must not fall much below 300 mm.^{3} and, on the other hand, the establishment of diffusion equilibrium is endangered, particularly in experiments in serum, by a more rapid gas exchange than about 100 mm.^{3} per hour. Hence, in general, the experiments lasted for about 3–5 hours; the figures given for respiration and glycolysis are, therefore, the mean values over the whole of this period.

Accuracy of the method.

Table I contains the results of a number of duplicate experiments by the new method, made simultaneously on different slices of the same piece of tissue or, in the case of embryo, by the use of two embryos of the same age. The Table shows that the accuracy of the method is sufficient and is in agreement with that to be expected from the considerations given in the previous paper.

Table I.

Tissue	Serum or Ringer solution	R.Q.
Liver	S	0·72, 0·71
	S	0·73, 0·71
Kidney	R	0·81, 0·82
Testis	R	0·88, 0·87
	R	0·93, 0·92
	S	0·93, 0·92
Brain cortex	R	0·99, 0·99
Chicken embryo	R	0·99, 0·98
Jensen sarcoma	R	0·75, 0·73
	S	0·79, 0·79
Slow-growing sarcoma	S	0·77, 0·77

Results.

The results of all measurements are collected in Table II. The tissues selected are representatives of groups with differing degrees of anaerobic glycolysis. A typical protocol of an experiment, showing the details of an actual measurement and also the method of calculation, was given at the end of Part III.

R.Q. in bicarbonate and in phosphate. In a number of experiments simultaneous duplicate determinations of R.Q. were made on slices from the same

Table II. *Metabolism in bicarbonate and phosphate.*

Specimen no.	Tissue	Bicarbonate-medium: Ringer or serum	$NaHCO_3$ m-mols	CO_2 in gas mixture vols. %	R.Q.	Q_{O_2} (respiration)	$Q^{O_2}_{CO_2}$ (glycolysis)	Phosphate-medium: R.Q. (Same specimen of tissue as in bicarbonate)	Phosphate-medium: Q_{O_2} (Same specimen of tissue as in bicarbonate)
	Liver								
6	Fasted	R	14	1·3	0·55	13·4	2·9	—	—
7	Fed	R	14	1·3	0·86	9·7	2·1	—	—
9	Fasted 12 hrs.	R	14	1·3	0·77	10·7	1·4	0·71 0·68	7·6 6·4
22	Fasted	S	20	4·5	0·72 0·71	9·1 8·5	0·9 0·9	0·65 0·63	9·9 10·2
23	Fasted	S	20	4·5	0·73 0·71	12·7 11·2	1·3 1·6	0·58 0·58	8·6 9·5
35	Fed	S	20	4·5	0·76 0·73	10·0 9·3	2·3 2·6	—	—
36	Fasted	R	20	4·5	0·66 0·64 0·66	13·7 13·9 13·5	4·2 4·3 4·1	0·68 0·68	11·4 11·2
	Kidney								
1	—	R	14	3·2	0·82	18·9	0	—	—
2	—	R	14	1·9	0·78	19·1	0	—	—
18	—	R	25	4·5	0·81 0·82	22·1 20·7	0 0	0·85 0·82	20·7 18·0
19	—	S	20	4·5	0·89	22·8	−6·2	—	—
28	—	S	20	4·5	0·86	22·8	−3·2	0·86 0·87	23·0 26·0
	Testis								
5	—	R	14	—	0·87	10·2	2·8	—	—
12	—	R	25	—	0·88 0·87	8·5 7·3	4·3 4·6	0·88 0·87	7·7 8·6
20	—	R	25	—	0·93 0·92	10·0 10·5	4·9 5·5	0·89 0·91	9·3 9·1
21	—	S	20	—	0·93 0·92	8·4 9·1	2·8 2·6	0·96 0·93	8·5 7·9
	Brain cortex								
3	—	R	14	1·3	1·00	10·6	1·6	—	—
4	—	R	14	1·3	0·99	12·8	1·2	—	—
11	—	R	25	5·0	1·00	16·2	0·3	—	—
15	Young rat	R	25	4·5	0·99 0·99	14·0 14·8	2·5 2·1	—	—
16	Old rat	R	25	4·5	0·96 0·94	15·2 14·4	3·2 2·7	—	—
17	Young rat	S	20	4·5	1·04	13·9	(3·9)	—	—
29	Senile rat	R	25	—	0·95 0·95	10·0 10·0	2·7 3·2	—	—
	Chorion								
10	From embryo of dry wt. 145 mg.	R	25	4·8	0·98	25·4	7·4	—	—
25	From embryo of dry wt. 161 mg.	S	20	4·5	1·02	26·9	−0·9	—	—
	Retina								
31	—	R	20	4·5	1·00	19·5	26·9	—	—
32	—	R	20	4·5	1·03	18·8	19·7	—	—
	Embryo (at 40°. Chicken 4th day)								
33	(Dry wt. 4·10 mg.)	R	20	4·5	0·98	11·3	1·3	—	—
34	(Dry wt. 3·08 mg.)	R	20	4·5	0·99	11·6	3·2	—	—
	Jensen sarcoma								
13	—	R	25	4·5	0·75 0·73	9·5 9·0	8·9 8·9	0·80 0·78	8·5 8·0
26	—	R	25	4·5	0·84 0·77	10·1 10·0	10·4 16·0	—	—
27	—	S	20	4·5	0·79 0·79	8·0 8·8	9·6 11·6	0·79 0·79	8·7 11·1
	Slow-growing sarcoma								
30	—	S	20	4·5	0·77 0·77	5·6 4·3	9·8 7·0	0·88 0·90	5·5 6·3

piece of tissue suspended (*a*) in bicarbonate-Ringer or serum, and (*b*) in phosphate. The compositions of the bicarbonate-Ringer and phosphate-Ringer solutions used were identical in respect to glucose (0·2 %), calcium and potassium, and the concentration of bicarbonate was also equal to that of secondary phosphate. Such control experiments with phosphate-Ringer were not made with those tissues previously shown to have a R.Q. of unity in this medium (brain, embryo, retina, chorion).

Reference to these individual experiments, which are included in Table II, shows that with the exception of liver tissue (see below) there is no systematic difference in the values of R.Q. observed in the two types of media, nor any significant difference in R.Q. due to the use of either serum or Ringer solution, or to the different CO_2 tensions or bicarbonate concentrations shown in the Table. These facts are also fully supported by a comparison of the mean values of R.Q. These figures are given in Table III, in which the mean values obtained in the numerous experiments in Part II are compared with the mean of the figures in bicarbonate-media obtained in the present paper.

Table III.

Tissue	Bicarbonate-medium. Mean R.Q.	Phosphate-medium R.Q. Mean of all previous experiments
Liver	0·71	0·79
Kidney	0·83	0·85
Testis	0·90	0·94
Brain cortex	0·98	0·99
Embryo (chicken)	0·99	1·00
Chorion (rat)	1·00	1·02
Retina	1·01	1·00
Jensen sarcoma	0·78	0·83
Slow-growing sarcoma	0·77	0·94

From Table III it is clear that if the tissues are arranged according to the mean value of R.Q., not only the order of the various tissues in the series is the same, but in addition to this, in the case of each individual tissue, the values of R.Q. in phosphate and bicarbonate respectively differ only slightly. In all normal tissues the difference does not exceed the variation between the measurements on different specimens of the same tissue measured in the same medium. However, it may be significant that in all cases the mean R.Q. in bicarbonate is very slightly below that in phosphate.

In the two types of tumour examined this difference, though in the same direction, is more pronounced. The number of specimens examined as yet is insufficient for any general conclusion to be drawn from this fact, but it is sufficient to note that in all examples the R.Q. isdefinitely below unity, and is in fact lower in bicarbonate or serum than in phosphate.

R.Q. of liver tissue. In a number of experiments in phosphate-media values of R.Q. below the fat-level have been obtained with livers of fasted rats (Table IV). The time since last giving food was in most cases 24 hours. Water was present in the cages throughout. These low quotients are not observed

Table IV. *R.Q. of liver of fasted rats measured in phosphate-Ringer solution, 0·2 % glucose, 38°.*

R.Q.	Q_{O_2}
0·61	8·9
0·57	7·1
0·59	7·8
0·62	8·4
0·62	8·7
0·65	9·9
0·63	10·2
0·58	8·6
0·58	9·5

in the case of animals which have been fed during a period of about 12 hours preceding the experiment. Consequently, they were not encountered in the series of rats used for the experiments in Part II, since in these experiments all the animals were fed either on the day of the experiment or on the evening of the previous day.

When similar experiments with fasted and with fed animals were made in bicarbonate-Ringer solution and in serum, it was found that the measurements in Ringer solution resembled those in the phosphate-medium, in that values of R.Q. below the physiological level were obtained with fasting animals. In serum, on the other hand, the value of R.Q. was within the normal limits and corresponded with the value found for the oxidation of fats.

R.Q. of brain tissue. In some of the experiments with cerebral cortex of rats, quotients a little below unity were obtained (Table II). It was noted that in all these experiments old rats had been used; in one example (specimen 29), R.Q. = 0·95, a very old animal aged about 2 years was used. The results suggest a tendency for the R.Q. to fall a little below the carbohydrate level with advancing age of the rat in the case of brain tissue.

Measurements of respiration (Q_{O_2}) *by the new method.* Reference to the figures in Table V shows that the extent of respiration as measured by the direct method used in this paper is in agreement with that found by Warburg [1930] by the use of his bicarbonate method. In view of the fact that our

Table V.

Tissue	Q_{O_2} This paper (mean value)	Q_{O_2} Warburg [1927]
Kidney	21·1	21
Liver	11·3	12
Testis	8·4	12
Brain	13·2	11
Chorion	26·2[1]	14·4[2]
Retina	19·2	17[3], 31[4]
Embryo	11·5[5]	10[5]
Jensen sarcoma	9·2	9

[1] Embryo dry weight 150 mg.
[2] Embryo dry weight 15·4 mg. [Negelein, 1925].
[3] Warburg, Negelein and Posener [1924].
[4] Warburg, Posener and Negelein [1924].
[5] Chicken embryo, weights 3·5 and 1·7 mg. respectively (dried at 110°).

measurements extend over a period of 3–5 hours, and are therefore not maximal but mean values over this period, this agreement is quite satisfactory, and shows that in most cases the respiration remained nearly constant over this long period. In one instance, that of chorion, the respiration observed on two separate specimens was found to be considerably higher than that given by Negelein [1925]. The latter, however, refers to the membrane obtained from smaller embryos than those which provided the material for our experiments.

Measurements of glycolysis ($Q_{CO_2}^{O_2}$). The new method has the advantage over the phosphate method that it permits the simultaneous measurement of aerobic glycolysis, as well as respiration and R.Q., on the same piece of tissue. The method is the only one suitable for the detection of slight aerobic formation of acid, either in Ringer solution or serum. From Table II it will be seen that, under the conditions of these experiments, nearly all normal tissues produce small amounts of acid, and this is true whether the medium for the experiment be serum or bicarbonate-Ringer. It will be noticed, for instance, that in the experiment with testis the acid production diminishes but does not disappear completely even in serum.

The only exceptions to the above statement in the series of tissues studied, were kidney and chorion. With kidney in Ringer solution the bicarbonate content in all four experiments was, within the limits of experimental error, quite unchanged during the whole period of the experiment. In serum, on the other hand, kidney caused a definite increase in the content of bound carbon dioxide over and above that corresponding to the retention of respiratory carbon dioxide by the serum (specimens 19 and 28, Table II). In the case of chorion, the appreciable aerobic glycolysis found in Ringer solution not only disappears in serum [Negelein, 1925] but there is even a slight consumption of fixed acid in the latter medium (specimen 25).

It should be remarked that knowledge of the nature of the traces of acid produced aerobically is at present inadequate. Owing to the small amount of acid formed in such cases it is very difficult to control the manometric observations on this point by reliable chemical methods of analysis, and it cannot yet be definitely assumed that all the acid formed is lactic acid in the case of the feebly glycolysing normal tissues.

A further complication in interpreting these data for the aerobic glycolysis of normal tissues, is the possibility of a portion of the acid formed being neutralised by a simultaneous ammonia-liberation by the tissue. For instance, in the case of kidney even in the presence of glucose an amount of ammonia (0·9 mm.^{3} per mg. tissue per hour [Warburg, 1930]) comparable with the figures for aerobic glycolysis found in the other examples of normal tissues in Table II (*e.g.* in liver tissue), appears in the solution in which the kidney tissue is suspended.

Turning to the highly glycolysing tissues such as the retina and tumours, it will be seen from Table II that the figures for aerobic glycolysis are lower

than those given by Warburg's bicarbonate method. This is due to the extended period of the experiments and the consequent appreciable lowering of the bicarbonate content of the Ringer solution or serum.

It is not possible in such cases to combine maximum accuracy in determining R.Q. with determination of glycolysis under optimal conditions. In highly glycolysing tissues separate experiments of short duration, such as those of Warburg [1930], must be used for obtaining a standard value of $Q_{CO_2}^{O_2}$.

Discussion.

Effect of medium on respiration and R.Q.

Phosphate- and bicarbonate-Ringer solutions. As was described in Part II, the extent of the respiration of surviving tissues does not suffer when the bicarbonate buffer in the medium is replaced by phosphate. This fact has also been well recognised in work on isolated muscle [Meyerhof, 1930, p. 53], and it is only in the case of other tissues that the suitability of phosphate appears to be questioned. The experiments described in the present paper show that the R.Q. is also very nearly the same in bicarbonate-media as in the simple phosphate-buffered Ringer solution previously used. Hence the respiration is both quantitatively and also, at least as far as concerns the type of metabolites oxidised, qualitatively independent of the nature of the buffer solution in which the tissue is suspended. As far as the available evidence shows, the state of the tissue in these different media seems to differ only in respect of its behaviour under certain quite special conditions. A much discussed example of this is provided by the action of cyanide in the two media [Dixon and Elliot, 1929; Alt, 1930; Warburg, 1931].

Serum and Ringer solution. The evidence previously advanced in favour of the use of serum as a medium for measurement of tissue metabolism was the effect on the aerobic glycolysis [Warburg, 1930]. In our experiments there is in most cases a tendency for aerobic glycolysis to be diminished in serum (*e.g.* in the case of testis), a fact which may be interpreted as indicating some damage to the tissue in the simple salt solution. The best example is given by chorion in which the appreciable aerobic glycolysis observed in Ringer-bicarbonate disappears when the tissue is suspended in serum (specimen 25). A new instance observed in the present paper is the effect of serum on the abnormally low R.Q. observed with the livers of starved animals in simple salt solutions. Here the damage to respiration in Ringer solution is shown by a value of R.Q. which may be compared with that of the atypical type of oxidation found in mechanically damaged tissue and in tissue extracts. That the effect of serum in this case is not to be explained by consumption of lactic acid contained in normal physiological concentration in this medium is shown by the fact that in all three experiments in serum (specimens 22, 23, 35), there was no disappearance of fixed acid from the medium (see, for instance, protocol at end of Part III) either with fasted or with fed animals. The con-

centration of lactate in the serum, however, was sufficient to cause a marked consumption of this substance by kidney tissue in serum (specimens 19 and 28).

It is proposed to investigate more fully this question of the consumption of lactic and pyruvic acids by tissues, for which purpose the method used in this paper is particularly suitable. One of the problems awaiting solution is the discrepancy between the above findings and the utilisation of lactic acid by liver *in vivo*. *In vitro*, in the presence of a large concentration of lactate, there is a definite increase in the respiration with accompanying consumption of lactate [Meyerhof and Lohmann, 1926] and increase of the value of R.Q. [Dickens and Šimer, 1930] of liver tissue above the level in glucose. Meyerhof and Lohmann [1926] also observe an action of serum in increasing the respiration of liver tissue which they attribute to the same cause, namely the presence of lactate in the serum. Our experiments on this point do not support this view, since no disappearance of lactic acid was observed, at least from serum of normal lactic acid content and containing an adequate supply of glucose. The same authors give some low figures for R.Q. of the liver of a starved rat in serum. The serum used by them however was acidified and the CO_2 removed by evacuation.

Relationship between respiration and glycolysis. The values in Table III and the agreement of individual comparisons in phosphate and bicarbonate (Table II) are sufficient to establish the correctness of the values of R.Q. previously measured. All quotients not only in normal tissues, but also in tumours, lie between the limits corresponding to the complete oxidation of normal foodstuffs.

The regular relationship previously found between the oxidative and anaerobic metabolism of carbohydrate in normal tissues is confirmed by measurements made under physiological tension of CO_2 in serum or bicarbonate-Ringer solution. Under the same conditions the R.Q. of tumour tissue is definitely below the carbohydrate level, and indicates a damaged oxidation of carbohydrate by such tissue.

The relationship found for other normal tissues can also be extended to the case of resting muscle. The value of R.Q. is below the carbohydrate level in this tissue (*e.g.* for diaphragm of the mouse R.Q. = 0·84, mean of six experiments in phosphate Ringer with glucose), a fact which is in accordance with the value of anaerobic glycolysis ($Q^{N_2}_{CO_2} = 3$–4). Unfortunately, similar measurements on working mammalian muscle are not available. A more general illustration of this relationship is provided by the developing embryo. As was shown by Negelein [1925] the glycolytic power of embryonic tissues falls regularly during development. Our measurement of R.Q. of young (4–5 day) chicken embryos shows that the oxidative metabolism of such embryos is a purely carbohydrate one. Needham [1931] in his exhaustive discussion of the probable nature of foodstuffs utilised during embryonic development, regards the development of the chick embryo as being divided into three periods of which the first, corresponding to the first five or six days

of incubation, is a period of carbohydrate oxidation; the second a period of protein utilisation and in the last stage the energy is derived mainly from the oxidation of fat. Corresponding with the disappearance of free glucose in the first eight days, an accumulation of lactic acid was found by Tomita [quoted by Needham, 1931]; this subsequently falls to a low level. All these facts are in complete agreement with our general conclusion that the replacement of an oxidative metabolism of carbohydrate by one of fat is associated with a corresponding lowering of the glycolytic power; the oxidative and glycolytic cleavage of carbohydrate proceeding hand in hand in this as in other cases.

SUMMARY.

1. The method for the measurement of R.Q., respiration and aerobic glycolysis previously described (Part III) has been applied to a series of normal and tumour tissues.

2. The accuracy of the method is shown by the good agreement between duplicate determinations.

3. No significant difference has been observed in the values of R.Q. and respiration measured in salt solutions buffered (*a*) by bicarbonate under physiological tension of CO_2, or (*b*) by phosphate in a CO_2-free atmosphere.

4. The respiratory quotients of the tissues of fed animals are in all cases within the limits corresponding to the oxidation of normal foodstuffs.

5. In fasting animals, the R.Q. of liver tissue in simple salt solutions containing glucose falls below the physiological level. It is restored to the fat level when the measurement is made in serum.

6. The conclusions derived from the experiments in phosphate-media previously reported (Part II) are established for bicarbonate-media also, and some new examples of the relationship between oxidation of carbohydrate and its glycolytic cleavage are brought forward.

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CIX. GROWTH AND CHEMICAL COMPOSITION OF OVUM OF FUNCTIONING FOWL'S OVARY (*GALLUS DOMESTICUS*).

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THE rate of growth of the ovum of *Gallus domesticus* was divided by the earliest workers into several more or less distinct periods. His [1868] distinguished by microscopic appearance four periods, while D'Hollander [1904] distinguished, on the basis of nuclear phenomena, three periods. Later Riddle [1916] suggested that there might be only two phases of the growth of the ovum: (1) from primary oöcytes up to 3 mm. in diameter, and (2) from 3 mm. to 6 mm. in diameter. At the end of the second period the rate of growth of the ovum is marked by a rapid deposition of yolk. Within 5 to 8 days the ovum reaches full maturity and is ready for exclusion. The latter period of growth or maturation of the ovum is caused by rapid assimilation of food materials, which constitute the chemical elements of the yolk proper. These chemical elements have probably never been determined quantitatively in successive stages of maturation as well as during the early growth period of the ovum. To throw some light on this problem a series of analyses has been performed.

The ova used were only from White Leghorn hens of known history. The experiments were conducted in the spring months (May and June) when the ovaries of possibly all egg-laying animals are under natural stimulus for egg production [Hoffmann, 1892]. A hen was killed at the time when an egg could be detected in the lower portion (uterus) of the oviduct, to provide uniform conditions in respect to the size of all ova in the examined ovaries. The length of oviducts was found to be on an average 59 cm., which corresponds with the results of Buckner and Martin's [1929] study of laying hens.

The data on the growth of the fowl's ovum are shown in Table I. As already suggested, these indicate growth periods, shown by the weight and shape of the ovum. Riddle [1916] and Stieve [1918] demonstrated that the ovum is almost a spherical body. However, our data on the shape of ova have been obtained by measuring the height (with spherometer) and width (with micrometer) of an ovum laid on a plane surface. The ratio of height to width might therefore be different from that described by the above workers. It is an index of elasticity of the vitelline membrane of the ovum.

Table I. *Growth of fowl's ovum.*

Phases of growth	Series of ova No.	Size of ovum: Average weight g.	Size of ovum: Dry matter content %	Shape of ovum: Height mm.	Shape of ovum: Width mm.	Shape of ovum: Ratio H/W Index
After laying	—	17·650	52·36	14·5	39·0	0·37
Before laying	—	17·076	52·76	16·0	35·5	0·45
Maturation phase	1	15·029	54·54	23·5	32·5	0·72
	2	10·782	53·20	20·5	29·5	0·69
	3	5·572	51·05	17·0	23·5	0·72
	4	2·287	48·15	12·0	17·5	0 69
	5	0·608	39·30	7·5	10·5	0·71
	6	0·250	18·57	6·0	8·5	0·71
	7	0·199	17·60	5·5	7·5	0·73
Second phase of growth	8	0·169	15·18	5·25	7·0	0·75
	9	0·157	14·45	5·0	6·5	0·77
	10	0·144	14·25	4·25	5·5	0·77
	11	0·128	14·12	3·5	4·5	0·78
	12	0·122	13·47	3·25	4·0	0·81
	13	0·115	11·90	3·0	3·75	0·80
	14	0·095	14·43	3·0	3·5	0·86
	15	0·089	13·58	3·0	3·5	0·86
	16	0·083	11·32	3·0	3·5	0·86
	17	0·071	15·31	3·0	3·5	0·86
	18	0·065	13·31	3·0	3·5	0·86
	19	0·063	12·80	3·0	3·5	0·86
	20	0·058	—	3·0	3·5	0·86
	21	0·045	—	3·0	3·5	0·86
	22	0·042	—	2·75	3·25	0·85
First phase of growth	23	0·035	—	2·75	3·0	0·92
	24	0·016	—	2·75	3·0	0·92
	25	0·013	—	2·5	2·75	0·91
	26	0·012	—	2·5	2·75	0·91
	27	0·012	—	2·5	2·75	0·91
	28	0·012	—	2·5	2·75	0·91

This index is noticeably high in small ova, and rapidly declines after ovulation, because the yolk, by absorption of moisture from the surrounding albumin, enlarges in size, becomes watery and thus weakens the elasticity of the vitelline membrane. The percentage of dry matter rapidly increases during maturation, reaching its highest point in a large ovum. Soon after ovulation this value drops to that which is usually found in fresh eggs.

The density of the contents of large ova corresponds with the percentage

Table II. *Chemical composition of fowl's ovum.*

Series of ova	Density $\frac{W_1}{W} D$	Calcium (CaO): Content g.	Calcium (CaO): % of wet weight	Fat (ether extract): Content g.	Fat (ether extract): % of wet weight	Fat (ether extract): Iodine value (Wijs)	Fat (ether extract): Refractive index	p_H
After laying	1·03181	0·412	2·33	5·363	30·38	72·58	1·4685	5·985
Before laying	1·03187	0·410	2·40	5·148	30·15	(71·95)	1·4679	5·968
In ovary:								
1st ovum	1·03448	0·390	2·59	4·294	28·57	73·35	1·4685	5·900
2nd ,,	1·03372	0·208	1·93	3·161	29·32	68·33	1·4675	5·917
3rd ,,	1·03128	0·134	2·40	1·710	30·69	60·95	1·4679	6·027
4th ,,	—	0·091	3·98	0·652	28·51	—	1·4672	6·137
5th ,,	—	0·029	4·77	—	—	—	—	6·289

of dry matter (Table II). The content of calcium, as calcium oxide, gradually increases up to the time of ovulation with a slight change in the percentage composition. Fat (ether extract) content increases but without change in the percentage composition. There is an indication of a slight change in the iodine value, and no change whatsoever in the refractive index. Hydrogen ion concentration shows little if any change.

Conclusions.

The growth phases and particularly the maturation phase of the fowl's ovum are marked by a very rapid increase in the percentage of dry matter, reaching the highest point in a mature ovum.

Calcium and fat contents of the ovum increase proportionately with the advance of maturation.

Density and p_H of ovum, iodine value and refractive index of the extracted fat show only slight changes during the maturation phase.

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CX. A NEW SYNTHESIS OF METHIONINE.

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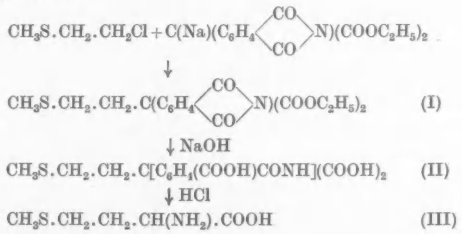
(*Received May 21st, 1931.*)

The Strecker synthesis of methionine by Barger and Coyne [1928] served its purpose in establishing the constitution of the amino-acid, but the yield (6 %, based on the β-methylthiolpropaldehyde employed) did not help to make the substance more easily available than the natural product, which is very troublesome to isolate. A second synthesis by Windus and Marvel [1930] (bromination of β-methylthiolethylmalonic acid) did not result in a greatly improved yield (7 % based on the α-methylthiol-β-chloroethane employed), probably owing to the oxidation of the methylthiol group.

In order to render the amino-acid more readily available for metabolic studies and for investigation of its optical properties, we thought it desirable to explore other possible syntheses with a view to obtaining increased yields. The synthesis described in this paper avoids the main difficulties of the preceding ones, namely the use of a rather inaccessible intermediate, acrolein, and the oxidation of the methylthiol group by bromination. By its use we have been able to accumulate a considerable supply of methionine and at present we are engaged in its resolution. It will be recalled that the method for the isolation of the naturally occurring substance involves the use of alkali under conditions which probably lead to at least partial racemisation.

Following the lines which led Sørensen [1903–6] to the synthesis of α-aminoadipic acid and other amino-acids, ethyl sodiophthalimidomalonate was made to react with α-methylthiol-β-chloroethane. On hydrolysis of the resulting compound (I), first with alkali to the phthalamidomalonic acid (II) and then with acid, methionine (III) was obtained in a yield of 58 % (based on the α-methylthiol-β-chloroethane employed). The reactions are represented by the following equations:



Experimental.

α-Methylthiol-β-chloroethane. 440 g. of methyl *iso*thiocarbamide sulphate, prepared according to the method of Arndt [1921] in a yield of 95 %, were hydrolysed by 670 cc. of 5*N* sodium hydroxide and the methylmercaptan obtained was directly absorbed in a solution of 5·5 g. of sodium in 1185 cc. of commercial alcohol. This solution was heated to boiling and 207 g. of ethylene chlorohydrin were added in such a manner that the solution continued to reflux gently. After the addition of the ethylene chlorohydrin the solution was boiled for 10 hours. It was found inadvisable to distil the alcohol from the solution since the large amount of sodium chloride present caused considerable danger of breakage from bumping. The solution was centrifuged and the salt shaken up with successive quantities of absolute alcohol until it retained no appreciable odour of the thiol compound. The alcoholic solution of the thiol compound was then distilled *in vacuo*, the yield of α-methylthiol-β-hydroxyethane being 126 g. of a product boiling at 59°–70°/16 mm. (51 % of the theory based on the ethylene chlorohydrin employed).

The α-methylthiol-β-hydroxyethane was chlorinated by the method of Bennett [1929]. 318 g. of thionyl chloride were dissolved in 700 g. of dry chloroform and the solution was added drop by drop to the dimethylaniline solution of the thiol compound. The solution was kept at room temperature for 2 hours and then heated for 20 minutes on the steam-bath. It was then washed three times with concentrated hydrochloric acid and twice with water, dried over calcium chloride, filtered, and freed from chloroform by distillation. The residue of crude chlorosulphide was distilled *in vacuo*. The yield was 115 g. of a product boiling at 50°/25 mm. (70 % of the theory).

Ethyl sodiophthalimidomalonate [Sørensen, 1905]. To a solution of 4·6 g. of clean sodium in 150 cc. of absolute alcohol (freshly distilled over sodium) at about 60°, 63 g. of ethyl phthalimidomalonate [Sørensen, 1903–6; Organic Syntheses, 1927] were added and the mixture was shaken. The sodium compound, which crystallised on cooling in ice-water, was filtered quickly at the pump, washed twice with absolute alcohol and twice with absolute ether and dried in a vacuum desiccator over sulphuric acid. The yield was 67 g., and by neutralisation of the mother-liquors with hydrochloric acid 5·5 g. of the ester were recovered. A sample of the sodium compound was dried to constant weight at 140°/10 mm., and in 6 hours lost 16·8 % of its weight (1½ molecules of alcohol of crystallisation require 17·43 % loss); the colour of the substance changed on drying from a rather deep orange-yellow to a very light yellow. This colour change can be used as an index of the completion of the drying of large quantities of the compound, which is essential if one wishes to obtain the highest yield possible in the subsequent step, and this was accomplished by heating in a bolt-head flask in an oil-bath at 145–155°/12 mm. with occasional shaking for 8 hours or until the change of colour of the substance to light yellow was complete.

(Found: 7·036 % Na. Calculated for $C_{15}H_{14}O_6NNa$: 7·04 % Na.)

In order to avoid the inconvenience of having to rid the substance of its alcohol of crystallisation, the method of Dunn and Smart [1930] may be used.

Ethyl β-methylthiol-α-phthalimidoethylmalonate (I). 85 g. of the ethyl sodiophthalimidomalonate were placed in a 250 cc. bolt-head flask fitted with a 3-holed rubber stopper for condenser, thermometer and stoppered glass tube for sampling; 43 g. of α-methylthiol-β-chloroethane (60 % excess) were added and the flask was heated in an oil-bath at 150° until the reaction mixture was no longer alkaline to turmeric paper (in this case the time was 1 hour and 45 minutes). The excess of the chloro-compound was distilled off *in vacuo* (after redistillation 12 g. of a pure product were obtained). The residual yellow oil was mixed with warm water and poured into an evaporating dish where it almost immediately crystallised. The crystalline substance was again extracted with water to remove any remaining sodium chloride and recrystallised from the smallest quantity of alcohol possible. It formed white prisms having M.P. 67° (uncorr.). The yield was 79 g. (74 % of the theory, based on the chloro-compound used).

(Found: 56·96 % C, 5·52 % H, 8·53 % S. Calculated for $C_{18}H_{21}O_6NS$: 57·14 % C, 5·58 % H, 8·44 % S.)

β-Methylthiol-α-phthalamidoethylmalonic acid (II). 25 g. of the ester were dissolved in 30 cc. of alcohol in a 200 cc. bolt-head flask, placed on a boiling water-bath and 70 cc. of 5*N* sodium hydroxide were added. The cloudy liquid was heated until a sample gave a clear solution when diluted with water. The sodium salt of the acid is insoluble in an excess of sodium hydroxide but very soluble in water. For the preparation of methionine it was unnecessary to isolate the free acid, though this was actually done. After the hydrolysis was completed the solution was cooled to 0° in an ice-salt mixture and carefully neutralised with 0·2 *N* hydrochloric acid to Congo red paper. To this solution, still at 0°, 75 cc. of 5*N* hydrochloric acid were added carefully, during which process the acid began to separate in the form of white crystals. In order to complete the salting-out 60 cc. of concentrated hydrochloric acid were added slowly. The white crystalline mass was then filtered at the pump and freed from salt by trituration with ice-cold water. The yield was 21·5 g. and 0·5 g. more was obtained by working up the mother-liquors (97 % of the theory). M.P. 142–143° (uncorr.).

(Found: 49·55 % C, 4·78 % H, 9·56 % S. Calculated for $C_{14}H_{15}O_7NS$: 49·27 % C, 4·40 % H, 9·38 % S.)

Methionine (III). 21·5 g. of the above acid were suspended in 350 cc. of water in a litre flask which was then placed on a boiling water-bath. 40 cc. of concentrated hydrochloric acid were added. Almost immediately carbon dioxide was given off and the substance began to go into solution. The heating was continued for about 1½ hours and 200 cc. more of concentrated hydrochloric acid were added which did not produce a further precipitate. Heating was continued for 45 minutes to complete the reaction. The water and acid were then removed by distillation first at ordinary pressure and then *in vacuo*.

The residue was taken up in warm water, 18 cc. of pyridine were added and three volumes of hot alcohol. On cooling the methionine separated as white crystals. The methionine was then suspended in 200 cc. of boiling absolute ether in order to remove any remaining phthalic acid, filtered at the pump and dried in a vacuum desiccator over sulphuric acid. M.P. 279°-280° (uncorr.). The yield was 8 g. (81 % of the theory based on the α-methylthiolphthalamidoethylmalonic acid employed, or 58 % of the theory based on the α-methylthiol-β-chloroethane).

(Found: 40·47 % C, 7·29 % H, 21·46 % S. Calculated for $C_5H_{11}O_2NS$: 40·23 % C, 7·43 % H, 21·50 % S.)

Methionine methyl ester hydrochloride. A suspension of 2 g. of methionine in 125 cc. absolute methyl alcohol, cooled in an ice-salt mixture, was saturated with hydrogen chloride for 2 hours. The alcohol and hydrochloric acid were then distilled off *in vacuo*. The residue was taken up in acetone from which it crystallised after 1 day in a vacuum desiccator. The ester hydrochloride was filtered off and washed with a small quantity of absolute ether. The yield was 1·9 g. (71 % of the theory) of white needle-like crystals. M.P. 135°.

(Found: 17·74 % Cl. Calculated for $C_6H_{14}O_2NSCl$: 17·59 % Cl.)

We gratefully acknowledge a grant from the Moray Fund of Edinburgh University which partially defrayed the expenses of this investigation.

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CXI. THE VITAMIN D PROBLEM.
II. OPTICAL ROTATION OF VITAMIN D.

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(*Received June 2nd, 1931.*)

The most simple reaction, that can take place when ergosterol is irradiated with ultraviolet rays, is, of course, Ergosterol → L, *i.e.* the ergosterol is transformed into one single substance, L.

In a previous paper [Reerink and van Wijk, 1929] we have been able to show that under certain conditions the reaction proceeds in such a way that the resulting phenomena are in full agreement with this reaction scheme. This was concluded from a study of the changes in the absorption spectrum the necessary conditions being:

(1) the light used for the irradiation must not contain rays of wave-length less than 284 $m\mu$[1]);

(2) the irradiation has to be stopped before more than about 50 % of the ergosterol has been transformed;

(3) oxygen must be completely absent;

(4) the solution must be vigorously stirred.

This result was of some importance, since the preparations obtained by irradiation of ergosterol under these conditions show intense antirachitic activity, the degree of activity being proportional to the degree of transformation of the ergosterol [Everse and van Niekerk, 1931, 1, 2]. Therefore, if indeed a single substance is formed under these conditions, this substance must be vitamin D. This conclusion being very important, we have tried to prove its correctness in an independent way, *viz.* by the measurement of the changes in the optical rotation on irradiation.

Experimental.

Since the complete absence of oxygen is a very important condition for the reproducibility of experiments on the irradiation of ergosterol, we have taken care to perform all operations *in vacuo*. In order to be able to do so, we have made extensive use of an implement well known in vacuum technique, the working of which will be easily understood from Fig. 1 *a*. In this way it is quite easy to connect an evacuated vessel with another one,

[1] Formerly we put the limit at 275 $m\mu$, but since then we have found it better to cut off at a somewhat greater wave-length [see Reerink and van Wijk, 1931].

which in its turn can be evacuated and sealed off from the pump; by breaking the partition-wall by means of an iron ball, operated magnetically, the communication between the two vessels is brought about. Thus it is possible *e.g.* to distil a liquid from one vessel into another, or to recrystallise a substance from a solvent and filter off the crystals with a sealed-in filtering disc of sintered glass *etc.*, all operations being performed under air-free conditions.

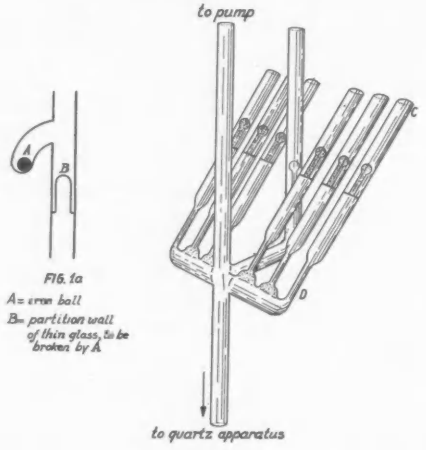

FIG. 1a
A = iron ball
B = partition wall of thin glass, to be broken by A

Fig. 1*b*.

The solutions were irradiated in an all-quartz apparatus, containing an electromagnetic stirrer and bearing a device for withdrawing samples. The working of the latter will be understood from Fig. 1 *b*: a small part of the solution to be examined can be decanted into one of the sample-tubes, *C*; the tube is then sealed off at the capillary part, *D*, and can be used for further examination. A polarimeter-tube was directly sealed to the apparatus.

The ergosterol had been recrystallised from alcohol-benzene [Bills and Honeywell, 1928] and proved to be quite pure, $[\alpha]_D^{18°}$ being $-134{\cdot}0°$ in chloroform. Pure peroxide-free ether was used as a solvent. The whole apparatus was thoroughly evacuated until the pressure of non-condensible gas was less than 0·001 mm., and then sealed off. The solution was irradiated with a quartz mercury arc through a filter of xylene-alcohol, cutting off all radiation below 284 $m\mu$. After a suitable period of irradiation, several readings of the rotation for the yellow sodium line were taken with a Schmidt and Haensch polarimeter; then one of the sample tubes was partially filled with solution and carefully sealed off from the rest of the apparatus. The degree of transformation of the ergosterol was estimated by the digitonin method in the sample withdrawn. With the rest of the solution the irradiation was continued, *etc.*

Experimental results.

(*a*) *Rotation.*

The results of three different series of experiments are shown in Table I, in which the first column gives the time of irradiation in hours, the second the % quantity of unchanged ergosterol as found with the digitonin method, and the third the specific rotation of the irradiated preparation.

Table I.

Time of irradiation in hours	% ergosterol	Rotation measured	Rotation calculated
0	100·0	−105·2°	.
1	93·9	−95·8	−94·5°
2	90·7	−87·5	−89·2
4	79·6	−71·3	−70·1
6	70·9	−56·9	−55·1
8½	60·2	−40·5	−36·8
0	100·0	−105·2	.
¼	96·8	−100·4	−99·5
3¼	77·7	−68·7	−66·9
0	100·0	−105·2	.
1	94·0	−94·3	−94·8
2	90·1	−85·2	−88·1
4	78·6	−70·0	−68·4
6	72·0	−55·3	−57·0
9	62·2	−38·4	−40·2
13	52·9	−21·5	−24·2

More illuminating is Fig. 2, where the measured values of the rotation from the three series of experiments are plotted against the relative amounts of unchanged ergosterol. This figure indicates clearly, that the points fall on

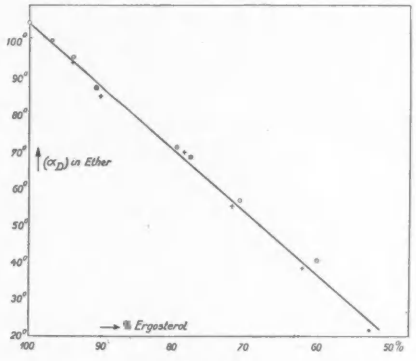

Fig. 2.

a straight line, the deviation being of the order of magnitude of the experimental errors. Now this behaviour is exactly what must be expected if the reaction-scheme of p. 1001 holds true.

If α_E and α_L are the specific rotations of ergosterol and of the reaction product, then, if x % of the ergosterol have been left unchanged, the measured rotation must be

$$\alpha_m = x.\alpha_E + (100-x)\,\alpha_L,$$

or

$$\alpha_m = x.(\alpha_E - \alpha_L) + 100.\alpha_L,$$

giving a linear relation between α_m and x.

Putting the experimental parameters of the line in Fig. 2, equal to the theoretical parameters of the above formula, we obtain for $\alpha_L = +\ 66{\cdot}7° \pm 2{\cdot}7°$ (in ether).

By means of this value for the specific rotation of the reaction-product, and of that of ergosterol, the corresponding rotation can be calculated for every degree of transformation (second column in Table I). These calculated rotations are shown in the fourth column of Table I; the deviation of their values from the experimental values prove to be within the limit of experimental error.

It may be concluded from the foregoing, that, just as in the experiments on the absorption spectrum, the experimental facts are in complete harmony with the assumption that the first long-wave reaction-product of ergosterol is a single substance. Still, as was already pointed out in an earlier paper, both series of experiments may also be explained by a more complex hypothesis, *viz.*, that several substances are formed simultaneously in a constant proportion. As up to the present in our experiments we have never found irrefutable facts that could not be explained by the former assumption, which moreover has the advantage of its very simplicity, we are inclined to consider the individuality of the substance L as practically certain.

(*b*) *Rate of the photochemical reaction.*

From the results of these experiments, an interesting calculation can be made concerning the velocity of the reaction: Ergosterol → L, under our experimental conditions.

Since the concentration of the solution is so high that practically all active radiation is absorbed, the rate of disappearance of the ergosterol would be constant, if the intensity of the radiation were kept constant and if the ergosterol were the only absorbent. Now for practical purposes the intensity of the radiation remains constant in one series of experiments, but the ergosterol is not the only absorbent, as the vitamin D, which is formed, also shows an absorption in the same range of wave-lengths. Under these circumstances the incident radiation is divided between the ergosterol and the vitamin, and we may safely assume that the ratio in which the radiation is absorbed by the separate substances is

$$I_{\text{erg}}/I_{\text{vit}} = (\epsilon_{\text{erg}}\, C_{\text{erg}})/(\epsilon_{\text{vit}}\, C_{\text{vit}}),$$

where I_{erg} and I_{vit} are the intensities of the radiation absorbed by ergosterol and the vitamin respectively, ϵ_{erg} and ϵ_{vit} are the absorption indices and

C_{erg} and C_{vit} the concentrations. Strictly, this equation holds for each wave-length present in the radiation used. From the absorption curves, given in our previous paper [1929], we can deduce, however, that for our purpose as an approximation, the average of ϵ_{erg} may be put equal to the average of ϵ_{vit} in the range of wave-lengths under consideration. Our equation then becomes:

$$I_{erg}/I_{vit} = C_{erg}/C_{vit}.$$

Since now

$$I_{total} = I_{erg} + I_{vit}$$

we have all the necessary data to calculate the rate of disappearance of the ergosterol:

$$-(d\,C_{erg})/dt = k.I_{erg}, \text{ in which } I_{erg} = C_{erg}/(C_{erg} + C_{vit}) \,.\, I_{total},$$

hence $-(d\,C_{erg})/dt = k.I_{total}.C_{erg}/C_{0\,erg}$[1] or $-(d\,C_{erg})/dt = k'\,C_{erg}$.

After integration, this equation leads to

$$\log.C_{erg}/C_{0\,erg} = k''.t, \text{ where } k'' = 2{\cdot}303\,k'.$$

We can now put this equation to the test with the data from Table I: this is done in Table II, where k'' is calculated for the two longer series of

Table II.

1	2	3	4	5	6
t	C_E/C_{E_0}	k''	t	C_E/C_{E_0}	k''
0	1·000	—	0	1·000	—
1	0·939	0·027[5]	1	0·940	0·027
2	0·907	0·021	2	0·901	0·023
4	0·796	0·025	4	0·786	0·026
6	0·709	0·025	6	0·720	0·024
8½	0·602	0·026	9	0·622	0·023
			13	0·529	0·021

measurements. From an inspection of the figures in the third and sixth columns of this table it can be concluded that the calculated factor k'' is remarkably constant in each series of experiments. This proves that our assumptions must have been fairly correct.

(c) *Crystalline preparation.*

There is still another indication for the purity of the preparations of the reaction-product obtained in this way, that is, the remarkable ease with which it is obtained in the crystalline state when the excess of unchanged ergosterol is removed by recrystallisation from ether and alcohol *in vacuo*. The procedure (including the precipitation of the residual ergosterol with digitonin) may be described somewhat more in detail.

An ethereal solution of ergosterol, *e.g.* 5 g. in 600 cc. is irradiated in the quartz apparatus during some hours, till a proper degree (*e.g.* 25 %) of transformation, as measured by the rotation, has been reached. The solution is

[1] In agreement with the assumption that only one substance is formed from the ergosterol, $C_{erg} + C_{vit} = C_{0\,erg}$, the ergosterol concentration at $t = 0$.

then transferred into another vessel, from which part of the ether is distilled. A large part of the unchanged ergosterol is then removed by cooling on ice and decanting the supernatant liquid into another vessel through a sintered glass-filter. The ether is then distilled off and replaced by about 75 cc. alcohol. By shaking vigorously the reaction-product is extracted from the crystalline mass along with only about 0·15 g. of ergosterol; then the solution is filtered into a new vessel, containing an alcoholic solution of digitonin, which precipitates the rest of the ergosterol. After filtering the liquid into a new vessel the alcohol is evaporated, and the reaction-product is extracted with ether. The ethereal solution again being filtered into another vessel the ether is distilled off; the ergosterol-free reaction-product is then obtained as a white crystalline mass, consisting of rosettes of fine needles. These crystals are very soluble in all ordinary organic solvents, but can be crystallised from strongly cooled alcohol or from diluted methyl or ethyl alcohol. Fig. 3 shows the crystals as they are obtained by evaporation of an ethereal solution.

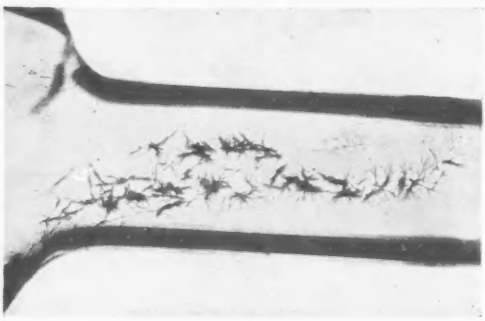

Fig. 3.

There is, however, one very interesting complication concerning this crystalline preparation, *viz.* the specific rotation is not 67°, as would be expected from the above measurements of the optical rotation of the primary reaction-product. In various experiments we have always obtained preparations with a rotation more or less exceeding the theoretical value. The longer the time between irradiation and final measurement of the rotation, and the higher the temperature used in the series of operations of recrystallisation and precipitation with digitonin or evaporation of the solvent, the higher was the value found for the rotation. For example, in one instance we found $[\alpha]_D = +94°$, in an experiment in which 20 days elapsed between irradiation and the final measurement, whilst the temperature had several times been as high as 50°. In another experiment, in which all operations had been performed as expeditiously as possible, and in which the temperature was kept as low as possible, the rotation was found to be + 68·1°.

This increase of the rotation even takes place when the solution in ether

is preserved on ice, as is shown in Table III, giving in 14 days an increase of about 7 %.

Table III.

Time	Rotation
0 days	+68·1°
3 „	+69·7
5 „	+71·1
14 „	+72·6

This shows that the crystalline substance is very unstable even in absence of oxygen and at a low temperature when it is dissolved in ether.

From the foregoing it follows that, though some of the physical properties of the pure vitamin can be derived comparatively easily from the properties of mixtures of vitamin D and ergosterol, other properties, for the measurement of which the vitamin D must be separated from the ergosterol, we cannot for the moment give with so much certainty.

We may, however, give some properties of one of our preparations from which the ergosterol had been removed as far as possible by means of several recrystallisations from alcohol.

Melting-point (in sealed evacuated tube) 115°–117°.

The ergosterol content of the preparation proved to be 3·7 %.

Specific rotation (corrected for ergosterol content): $[\alpha]_D = +100°$ (ether).

Analysis: H, 11·30 %, C, 85·4 %; calculated for $C_{27}H_{42}O$: H, 11·07 %, C, 84·8 %.

From the results of the analysis it may be concluded, that, during the very short time the preparation has been in contact with the air, no appreciable oxidation has taken place.

The crystals seem to be only very slightly hygroscopic, but in contact with the air gain rapidly in weight by oxidation, as is shown by the following experiment:

43·6 mg. were taken;

after 1 hour the weight was 43·7 mg.: increase 0·2 %.
„ 42 hours „ 45·5 mg.: increase 4·4 %.
„ 66 „ „ 45·9 mg.: increase 5·3 %.
„ 114 „ „ 46·4 mg.: increase 6·4 %.

In the meantime the colour had changed from pure white to bright yellow.

That this gain in weight is not a result of absorption of water follows from the fact that it is not reversed in a high vacuum.

Even when the preparation is kept in a thoroughly evacuated desiccator and brought into contact with air only during some brief interval necessary for taking out parts of it, it acquires a slight yellow tinge within a week and becomes oxidised.

Analysis: crystals, kept *in vacuo* for 1 week: H, 10·98 %, C, 83·5 %. Crystals kept in air for 114 hours: H, 10·37 %, C, 80·1 %; calculated: H, 11·07 %, C, 84·8 %.

This proves that the crystals are very susceptible to oxidation.

The absorption spectrum of the vitamin D after preservation is not the same as that of the pure vitamin D; as we have not yet found a definite form of absorption spectrum that is common to all such preparations, we will not for the present go further into this question.

In our former paper [1929] we mentioned the fact that vitamin D can be obtained in a crystalline state. These crystals were obtained by rapidly evaporating and thus cooling an ethereal solution; they showed a melting-point below 0°. The same phenomenon can be observed if an ethereal solution of the crystalline preparation described in this paper is treated in the same way. Thus it may be concluded that the crystals mentioned in the former paper were of the same substance as the present ones, the difference in melting-point being caused by ether of crystallisation.

Discussion.

(*a*) *Rotation.* The data which are found in the literature concerning the rotation of reaction-products of ergosterol show a wide variation. Whereas the earlier work of Windaus *et al.* [1929] and of Castille and Ruppol [1929] seems to indicate that the photochemical reaction-products show a relatively small rotation, Lahousse and Gonnard [1929] find for their most potent products a value for $[\alpha]_{578m\mu}$ of + 25° (alcohol).

In a recent paper, Windaus [1930] gives the rotation of a large number of ergosterol-free reaction-products; he states that his most potent samples prepared by irradiation with the Mg-spark show a low negative rotation; a high positive rotation was found for samples prepared with long-wave irradiation (Hg-arc and Uviol-glass filter), and for aged, or heated and over-irradiated samples. The dextrorotatory, long-wave irradiation products, which we would expect to be most similar to our preparations, proved to be less active than those prepared by irradiation with the Mg-spark. The difference between the results of Windaus and our own may be due to the fact that his preparations were mostly long-time irradiation-products (*ca.* 80 % of ergosterol transformed) whereas in our experiments the degree of transformation did not exceed 50 %.

(*b*) *The crystals.* Besides the crystals mentioned in our former publication, crystalline reaction-products from irradiated ergosterol have been described by Windaus [1930; Windaus *et al.* 1930] and by Askew *et al.* [1930, 1, 2]. Whereas the crystals described by Windaus are produced by over-irradiation and consequently do not show antirachitic activity, the crystalline preparation of Askew *et al.* shows a very intense activity and therefore consists at least partly of vitamin D. The melting point as well as the form of the crystals and the absorption spectrum suggest a similarity between these crystals and ours.

Since writing the above, we have had the privilege of discussing the subject with the English authors, who kindly informed us that their further purified crystalline preparation shows a much higher dextrorotation than ours; this

proves that the substance must be different. As to the nature of our crystals, we already pointed out that they probably do not consist of pure vitamin D, since the rotation of the crystalline preparation does not as a rule tally with the calculated rotation for pure vitamin D.

The way in which Askew *et al.* have obtained their crystalline preparation, *viz.* by distillation *in vacuo* of the crude reaction-product, implies the necessity of heating the vitamin D to temperatures above 100°; therefore in these experiments too the vitamin D is likely to undergo a change of the same nature as that occurring in our method of preparation.

The instability of vitamin D on storage or on heating has been described by Windaus [1930; Windaus and Auhagen, 1931] and by Askew *et al.* [1930, 1]. Though in some respects our results are not very different from those of these authors, there still exist a good many points in which they differ. Since, however, the experiments have been performed with reaction-products which may show a good deal of dissimilarity, the difference in the results does not at the present time provide a useful starting-point for a discussion. We wish to emphasise however one point of difference, *viz.* that, contrary to the experience of the other authors, we have never found an increase in absorption if the ergosterol-free reaction-product was irradiated with short-wave ultra-violet rays [Reerink and v. Wijk, 1931].

Summary.

Experiments are described in which the change of the rotation of ergosterol solutions on irradiation with long-wave ultra-violet rays is measured.

If the necessary precautions are taken, this change is very simple, the rotation showing a linear dependence on the degree of transformation of the ergosterol. These experiments provide further proof for the hypothesis, put forward in our previous publication, *viz.* that under definite conditions long-wave irradiation of ergosterol gives rise to one reaction-product only, which, on account of its intense antirachitic activity, must be vitamin D.

From these experiments the rotation of vitamin D can be calculated to be

$$[\alpha]_D = + 66{\cdot}7° \pm 2{\cdot}7° \text{ (in ether)}.$$

From correctly irradiated solutions, crystalline ergosterol-free preparations of vitamin D may easily be produced by recrystallisation and treatment with digitonin *in vacuo*. The properties of these preparations vary with the time and temperature used during the performance of the necessary operations, as a result of the instability of the vitamin D.

In conclusion we wish to express our thanks to Mr W. Breen and Mr J. Freen for their valuable assistance in the performance of the experiments.

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CXII. THE OXIDATION OF HEXOSE-DIPHOSPHORIC ACID BY AN ENZYME FROM ANIMAL TISSUES.

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Thunberg [1929] described the preparation from cucumber seeds of an enzyme which, in presence of methylene blue, was capable of oxidising citric acid. Of a very large number of possible substrates tested, hexosediphosphoric acid was the only substance other than citric acid which in dilute solution was oxidised by the enzyme preparation. A short time earlier, Bernheim [1928] described the preparation of an enzyme from liver which, in the presence of methylene blue, brought about the oxidation of citric acid. It seemed therefore of interest to see whether this enzyme preparation from liver could, like that from cucumber seed, also oxidise hexosediphosphate. The following experiment shows that hexosediphosphoric acid is indeed oxidised by the enzyme prepared from liver.

Acetone-liver was prepared from fresh ox liver by the method described by Bernheim [1928]. 30 g. of the dry preparation were extracted with 100 cc. of distilled water in a large mortar, the mixture being ground at intervals for $2\frac{1}{2}$ hours. After straining and squeezing through muslin, the residue was again ground for a few minutes with 50 cc. of distilled water and squeezed through muslin. The combined extracts were centrifuged, and the cloudy solution obtained was saturated with ammonium sulphate and allowed to filter overnight in the ice-chest. The precipitate was dissolved in phosphate buffer, the volume being made up to 45 cc., and was brought to p_H 7·6 with dilute sodium hydroxide and centrifuged to remove insoluble protein.

The sodium hexosediphosphate solution was prepared from a pure sample of calcium hexosediphosphate (calcium fructo-furanose-1:6-diphosphate) obtained from Bayer Products, Ltd. A weighed amount was dissolved in water, treated with very slightly less than the equivalent amount of pure sodium oxalate and centrifuged. After testing to ensure freedom from oxalate, the solution was stored below 0°, fresh solutions being made up at frequent intervals.

Exp. 1. The experiment was carried out using Thunberg vacuum tubes which were evacuated at the water-pump, filled with nitrogen, re-evacuated and put into a water-bath at 37°. All experiments described in this paper were carried out at this temperature. Each tube contained 1·5 cc. enzyme, 0·5 cc. phosphate buffer p_H 7·6, and 0·25 cc. methylene blue (1 in 5000).

Substrate added	Time for decoloration of methylene blue mins.
None	45
0·0125 cc. *M*/5 Na hexosediphosphate	21
0·025 cc. ,,	20
0·0125 cc. *M*/5 Na citrate	17
0·025 cc. ,,	15

A dialysed enzyme preparation made exactly according to Bernheim's method similarly oxidised hexosediphosphate. A solution of sodium hexosediphosphate prepared by acting on the barium salt (obtained from another source) with sodium sulphate gave similar reduction times with methylene blue. By precipitating the enzyme twice with ammonium sulphate the control time is lengthened, and the acceleration due to hexosediphosphate becomes relatively much greater, as is seen in Exp. 4.

Having shown that the hexosediphosphoric enzyme is associated with the citric enzyme not only, as shown by Thunberg, when prepared from cucumber seeds, but also when prepared from liver, an experiment was carried out to determine whether the oxidation of both substrates was brought about by the same enzyme. If one of the substrates be added to the enzyme in such amount that further addition causes no increase in the rate of reduction of methylene blue, the enzyme surface may be regarded as being saturated with the substrate. The further addition of the other substrate may produce one of two effects. If the same enzyme is responsible for the oxidation of both substrates, the two substrates will compete for the active surface of the enzyme, and the time of reduction of methylene blue with the mixture will be between the reduction times of the two substrates alone. On the other hand, if the oxidation of the two substrates is brought about by two different enzymes, when both substrates are present the two reactions will be occurring simultaneously on different enzyme surfaces, and the resulting rate of reduction will be greater (*i.e.* the time of reduction of methylene blue will be less) than that with either substrate alone. The following experiment shows that in the case of citric acid and hexosediphosphoric acid the latter alternative occurs and that the two substrates are therefore oxidised by different enzymes.

Exp. 2. Each vacuum tube contained 1·5 cc. enzyme + 0·5 cc. phosphate buffer p_H 7·6 + 0·25 cc. methylene blue. Water was added to make the total volume in each tube 2·45 cc.

Substrate added	Time for decoloration of methylene blue mins.
None	39
0·05 cc. *M*/10 Na hexosediphosphate	18
0·1 cc. ,,	17½
0·05 cc. *M*/10 Na citrate	12
0·1 cc. ,,	14
0·05 cc. *M*/10 Na hexosediphosphate + 0·05 cc. *M*/10 Na citrate	8½
0·1 cc. *M*/10 Na hexosediphosphate + 0·1 cc. *M*/10 Na citrate	7

The values for the mixed substrates agree fairly closely with the values calculated on the assumption that both reactions take place independently. This suggests that neither substrate is appreciably adsorbed on the enzyme which oxidises the other substrate, since such adsorption would be expected to produce an inhibition in the rate of oxidation [Quastel and Wooldridge, 1927].

An attempt was made to prepare the enzyme from rat muscle. 8 g. of fresh rat leg muscle was finely chopped and extracted with three times its weight of distilled water for $2\frac{1}{2}$ hours. The extract was strained through muslin and precipitated by saturation with ammonium sulphate. The precipitate was pressed on filter-paper, made up to 5 cc. in phosphate buffer and brought up to p_H 7·5 with dilute NaOH.

Exp. 3. Each vacuum tube contained 1 cc. enzyme + 0·25 cc. phosphate buffer p_H 7·5 + 0·125 cc. methylene blue.

Substrate added	Time for decoloration of methylene blue mins.
(0·1 cc. water)	130
0·1 cc. *M*/25 Na hexosediphosphate	40
0·1 cc. *M*/25 Na citrate	90

An enzyme solution can thus be extracted from rat muscle showing an activity towards hexosediphosphate similar to the activity of the enzyme extracted from an equal weight of ox liver. While, however, enzyme preparations from the latter usually show an approximately equal activity towards equimolecular solutions of citrate and hexosediphosphate, the enzyme solution from rat muscle contains relatively little citric dehydrogenase. Preparations have been obtained from rat muscle showing considerable activity towards hexosediphosphate and practically no activity towards citrate. Incidentally, this affords confirmation that the activation of the two substrates is brought about by different enzymes.

A similar preparation from fresh young rabbit leg muscle showed some activity towards hexosediphosphate but hardly any towards citrate. Other tissues have not so far been tested for the presence of the enzyme.

Experiments carried out with the Barcroft differential manometer at 37° indicate that while the enzyme solution by itself shows a small residual oxygen uptake, the addition of hexosediphosphate does not produce any appreciable increase in the oxygen uptake. If, however, methylene blue be added, a definite increase in oxygen uptake is observed, as would be expected, since the leuco-methylene blue formed by reduction of methylene blue takes up oxygen spontaneously. The inability of the enzyme to bring about direct oxidation of hexosediphosphate with oxygen does not in any way indicate that such an oxidation does not occur in the body. In the intact cell, there is good reason to believe [Keilin, 1929] that through the agency of cytochrome and the intracellular oxidase, active oxygen is made available for the oxidation of substrates of such dehydrogenase systems as the present one. That such oxidations can occur is shown in the next paper [Harrison, 1931].

Various workers have shown the presence of an enzyme in tissues capable of oxidising glycerophosphoric acid, and this substance was therefore tested as being a possible substrate for the hexosediphosphate dehydrogenase.

Exp. 4. Each vacuum tube contained 1 cc. enzyme solution (twice precipitated by saturation with ammonium sulphate) in phosphate buffer at p_H 7·4 and 0·33 cc. methylene blue.

Substrate added	Time for decoloration of methylene blue mins.
0·07 cc. water	77
0·07 cc. *M*/25 Na glycerophosphate	68
0·07 cc. *M*/25 Na hexosediphosphate	17

This experiment has been repeated a number of times with similar results. The glycerophosphate appears therefore to be oxidised to a very slight extent by the enzyme preparation, but the rate of oxidation is negligible compared with that of hexosediphosphate.

Properties of the enzyme preparation.

Both the citric and hexosediphosphoric dehydrogenases are only partially precipitated by half-saturation with ammonium sulphate, though half-saturation appears to bring down a greater proportion of the hexosediphosphoric enzyme than of the citric enzyme. Thus, although a large part of the hexosediphosphoric enzyme remains in the filtrate, a preparation of this enzyme may be obtained with the citric acid enzyme partially removed, and with the additional advantage of showing only a very small residual reducing power towards methylene blue in the absence of substrate.

If the liver extract be dialysed overnight in the ice-chest in a collodion sac, then acidified to p_H 5·7 and centrifuged before precipitation with ammonium sulphate, a perfectly clear solution of the enzyme can be obtained. This clear solution retains its activity even after filtration through kieselguhr or through a porcelain candle (Pasteur-Chamberland L 5).

While the hexosediphosphate enzyme present in the dry acetone-liver preparation is fairly stable if kept *in vacuo*, the solution of the enzyme is rather unstable and loses some of its activity even on standing overnight in the ice-chest. A solution of the enzyme in water at p_H 7·2 lost about one-third of its activity after being heated to 45° for half an hour. A neutral solution of the preparation brought to about p_H 4 with HCl, allowed to stand a few minutes and again neutralised, was not appreciably reduced in activity. After some hours at this p_H, however, the activity towards both citrate and hexosediphosphate is completely lost. The oxidation of hexosediphosphate falls off rapidly on the acid side of neutrality. The activity in phosphate buffer is much less at p_H 6 than at p_H 7. It is somewhat greater at p_H 8 than at p_H 7. Owing, however, to the loss of ammonia from the alkaline solutions with consequent change of p_H during evacuation of the tubes, and owing to the fact that the

reaction is strongly inhibited by borate and phthalate buffers, a satisfactory p_H-activity curve has not so far been obtained.

The optimum substrate concentration for the hexosediphosphate dehydrogenase at p_H 7·6 appears to be about $M/450$ and is of the same order as that for the citric dehydrogenase.

Since citric acid and hexosediphosphate are oxidised by different enzymes, it seems remarkable that these two enzymes should occur together relatively free from other enzymes when prepared from sources so different as cucumber seed and ox liver. The two enzymes show a considerable similarity in their physical properties as is shown by the lack of success of a number of attempts to separate the two by warming and by treatment with various adsorbents. It seems likely that the centres responsible for the two activations are closely associated on the same large protein molecule, and that this protein largely determines the physical properties of the enzymes.

No evidence has so far been obtained as to the nature of the product or products of this oxidation of hexosediphosphoric acid, and one can only speculate on the function of the enzyme in the body. Meyerhof [1930] has shown that during glycolysis in muscle, hexosediphosphate is built up, and in view of the high concentration of the hexosediphosphate dehydrogenase which I have shown to be present in muscle, it seems possible that the oxidation of hexosediphosphate takes place side by side with that of lactic acid during the recovery period in muscle.

Summary.

1. An enzyme (dehydrogenase) has been obtained from liver and muscle capable of oxidising hexosediphosphoric acid in presence of methylene blue.

2. In spite of the association of this enzyme with a citric acid dehydrogenase both when prepared from cucumber seed and from ox liver, the two enzymes are shown nevertheless to be distinct.

The author wishes to express his thanks to Prof. E. Mellanby for his interest in this work.

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CXIII. GLUCOSE DEHYDROGENASE: A NEW OXIDISING ENZYME FROM ANIMAL TISSUES.

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The preparation and properties of an enzyme which oxidises hexosediphosphate have already been described [Harrison, 1931]. During the course of this work, experiments were carried out to see whether the oxidation of hexosediphosphate was affected by the addition of other substances. Substances similar in structure to hexosediphosphate might, from the work of Quastel and Wooldridge [1928], be expected to be adsorbed at the same enzyme surface and so inhibit the oxidation. One of the substances tested was glucose, and it was surprising to find that this substance produced not an inhibition but a marked acceleration. Further investigation showed that there is present in the hexosediphosphate enzyme preparation another enzyme capable, in the presence of a suitable hydrogen acceptor, of bringing about the oxidation of glucose. Since an enzyme which oxidises glucose has not apparently been hitherto obtained from animal tissues, it seemed desirable to study its preparation and properties in some detail. It has been found possible to obtain perfectly clear solutions of the enzyme capable of being filtered through a porcelain filter without loss of activity.

Experimental.

Preparation of the enzyme.

Acetone-liver was prepared by a modification of the method described by Bernheim [1928]. About 1½ lbs. of fresh ox liver (obtained a few hours after the animal was killed) were weighed after peeling off the surrounding membrane, washed for about a minute under the cold tap, squeezed and minced twice through an ordinary fine mincer. The pulp was well stirred for about a minute with pure acetone (B.P.), using 900 cc. to 675 g. of liver, and filtered on a large Büchner funnel. The acetone treatment was repeated twice more, using half the above quantity of acetone for each extraction. After partially drying on the funnel for a short time, the product was spread out in two large desiccators over sulphuric acid and evacuated at the water-pump for several hours until most of the acetone was removed. The preparation is fairly stable if kept evacuated in the desiccator. In order to extract the enzyme, 30 g. of the

dry acetone-liver were put into a mortar with 120 cc. of distilled water and ground at intervals for 2 to 3 hours. The mixture was squeezed through coarse muslin, the residue ground up with 60 cc. of distilled water, squeezed through muslin, and the combined extracts were centrifuged for a short time. The opaque liquid was poured off (through muslin to remove the fatty layer which sometimes forms), put into thin collodion sacs, and dialysed overnight in the ice-chest against 2 litres of distilled water. The solution was then brought to about p_H 5·7 to bromocresol purple by the addition of about one drop of *N* HCl and the heavy protein precipitate centrifuged off. The clear or slightly opaque pigmented solution was brought approximately to neutrality by the addition of a few drops of *N* NaOH and was saturated with pure ammonium sulphate. After carefully pouring off from the excess of ammonium sulphate, the precipitate was filtered in the ice-chest on a starch-free filter-paper (Whatman, No. 40), partially dried by transferring to an absorbent porous tile, and finally removed from the plate and dried *in vacuo* over sulphuric acid. The enzyme, which is fairly stable in the dry form, was dissolved in water or phosphate buffer, made up to the required volume (30 cc. or 45 cc. are convenient volumes for methylene blue experiments) and brought to p_H 7·4 with *N* NaOH. This solution which is pigmented, but as a rule perfectly clear, was used for most of the experiments described in this paper. If the enzyme is required free from ammonium sulphate, the dry preparation is put into a wet collodion sac and dialysed against frequent changes of distilled water in the ice-chest until practically free from ammonium sulphate, after which it is centrifuged, a considerable amount of protein being thereby removed. The solution may then be concentrated by bringing to about p_H 7·8 with NaOH and distilling *in vacuo* in a large flask, keeping the temperature of the liquid below 25° and cooling the receiver in ice. The flask is heated on a water-bath at 30–35°. Frothing is sometimes troublesome at the beginning of the distillation, and it is advisable to allow a very slow stream of air (one bubble every few seconds) to bubble through a wide capillary under the surface of the liquid. If dialysis has been complete, the solution remains alkaline at the end of the distillation, and there is practically no destruction of the enzyme. The enzyme does not keep well in solution and loses some of its activity even on standing overnight in the ice-chest.

In preparing the enzyme, the following points should be noted. The temperature during the acetone treatment should not be much above 20°, and in hot weather, the acetone and the liver should be first cooled in the ice-chest. The acetone treatment should be carried out as rapidly as possible, and prolonged evacuation of the desiccators is desirable in order to remove the acetone as completely as possible. The collodion sacs used for dialysis should be kept sterile and should be renewed after using about three times, as they tend to become impermeable. In drying the enzyme, the thick absorbent porous plate is much preferable to the ordinary unglazed dinner plate. (The "Owens College" pattern from Griffin and Tatlock, Ltd., was found satisfactory.) The use of

ammonium sulphate in fairly large crystals facilitates decantation of the precipitated enzyme from the excess of ammonium sulphate. Starch-free filter-paper should always be used (except in preparing the acetone-liver) since the liver extract may break down starch to glucose.

The activity of the enzyme prepared from different samples of liver varies considerably, and occasionally an almost inactive preparation is obtained. The samples of liver which are soft and mince to a dark almost liquid pulp usually give the most active enzyme preparations. The enzyme has been prepared from the liver of the ox, sheep, cat and dog. Other tissues have not yet been examined.

Anaerobic oxidation of glucose.

The enzyme solution by itself usually shows a slight reducing power towards methylene blue varying considerably with different preparations. On adding glucose, the rate of reduction of methylene blue is greatly increased, as is shown in the following typical experiment. The methylene blue experiments were carried out at 37° in Thunberg vacuum tubes as previously described [Harrison, 1931]. 1 cc. of enzyme solution equivalent to 1 g. of acetone-liver was used in each tube together with 0·5 cc. phosphate buffer p_H 7·4 and 0·5 cc. methylene blue (1 : 5000). The glucose used was pure dextrose (A.R.), from British Drug Houses. The solutions of glucose were stored below 0° and fresh solutions were made up at frequent intervals.

Substrate added	Time of decoloration of methylene blue
None	28 mins.
0·05 cc. 2 *M* glucose	5 mins.

That the acceleration is due to glucose itself and not to an impurity is clear from the fact that different samples of pure glucose from different sources in suboptimal concentrations gave, with the same enzyme preparation, identical reduction times.

Pure fructose is not oxidised by the enzyme, and in the same concentration as glucose in the above experiment retards the residual reducing power of the enzyme preparation itself. Galactose and arabinose showed no appreciable reduction of methylene blue except in high concentrations, and it is not unlikely that the slow reduction which then occurred was due to traces of glucose present as an impurity in the sugars.

The effect of increasing the glucose concentration is shown in Fig. 1. The experiment was carried out at 37° using 1·0 cc. ammonium sulphate-free enzyme (equivalent to 1 g. acetone-liver) and 0·5 cc. methylene blue. The p_H was 7·6, and water was added where necessary to bring the total volume to 2·0 cc.

The optimum concentration of glucose is seen to be 0·25 *M* and in concentrations greater than this the velocity falls off slowly. The substrate concentration giving half the maximum velocity is about 0·07 *M* which is an exceptionally high value for an oxidising enzyme.

Lactic, citric and hexosediphosphoric acids are oxidised, though the optimum rates of oxidation with these substances are considerably smaller than that of glucose. Since, however, lactic acid is oxidised by the preparation, it was possible that the oxidation of glucose might not be a direct one, but that the glucose might first be glycolysed to lactic acid and the lactic acid then oxidised. That such is not the case is shown by the following experiment.

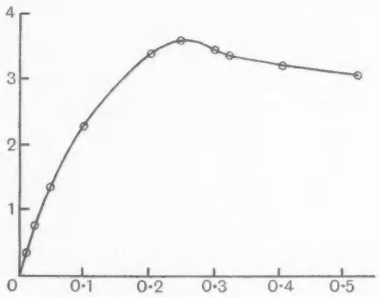

Fig. 1. Rate of glucose oxidation as a function of substrate concentration. Abscissa: molar concentration of glucose. Ordinate: velocity $= 100/T$, where T = methylene blue reduction time in minutes.

Four small flasks *A*, *A'*, *B*, *B'*, each contained 10 cc. of enzyme solution. To duplicates *B* and *B'* were added 3·0 cc. *M* glucose, while the duplicates *A* and *A'* received 3 cc. water. The flasks were then filled with nitrogen, tightly stoppered and incubated at 37° for $2\frac{1}{2}$ hours. 5 cc. of 20% trichloroacetic acid were then added to each flask, the solutions were filtered, glucose removed by the copper-lime method and lactic acid was estimated on an aliquot portion of each solution by the method of Friedemann, Cotonio and Shaffer [1927].

Flask	Mg. lactic acid
A	0·94
	= 0·90
A'	0·87
B	0·72
	= 0·73
B'	0·74

It is clear that there is no formation of lactic acid from the added glucose. Methylene blue experiments carried out at the same time with the same enzyme gave the following results. Each tube contained 1 cc. enzyme + 0·25 cc. methylene blue (1 in 5000) together with sufficient water to make the volume 1·55 cc.

Substrate added	Time for decoloration of methylene blue mins.
None	50
0·3 cc. *M*/1 glucose	5
0·1 cc. *M*/10 lactate	25
0·05 cc. *M*/10 hexosediphosphate	19
0·05 cc. *M*/10 hexosediphosphate + 0·1 cc. *M*/10 lactate	14
0·05 cc. *M*/50 hexosediphosphate + 0·1 cc. *M*/50 lactate	27

This shows that an amount of lactic acid much greater than the experimental error in the estimation of lactic acid produces only a relatively small acceleration in the rate of reduction of methylene blue.

The experiments on the mixture of hexosediphosphate and lactate were carried out, since the glucose enzyme preparation also contains an enzyme capable of oxidising hexosediphosphate, and Meyerhof and Lohmann [1927] have given evidence that in the lactic acid production in muscle extract with glucose, one molecule of hexosediphosphoric acid is formed with every two molecules of lactic acid. Mixtures of hexosediphosphate and lactate in the ratio of one molecule of the former to two of the latter in concentrations of $M/50$ and $M/10$ were found to be oxidised much more slowly than glucose. These results, coupled with the fact that no lactic acid could be detected on incubating the enzyme with glucose anaerobically, show that the oxidation of glucose by the enzyme is not due to the formation and oxidation of lactic acid either alone or with hexosediphosphoric acid. Indirect evidence in favour of this conclusion is furnished by the fact that neither sodium fluoride nor iodoacetic acid, both of which inhibit lactic acid formation in muscle, produces any inhibition in the rate of reduction of methylene blue by glucose *plus* the enzyme. (The experiments were carried out with $N/100$ sodium fluoride and with $N/1000$ sodium iodoacetate, both at p_H 7·6.)

A mixture of glucose and hexosediphosphate, the latter being above its optimum concentration, reduced methylene blue more quickly than either of the substrates in the same concentration by themselves. By the line of reasoning previously employed in proving the non-identity of the citric and hexosediphosphate enzymes [Harrison, 1931], this indicates that the oxidation of glucose and hexosediphosphate is brought about by two different enzymes, rather than by the same enzyme acting on two different substrates.

Properties of the enzyme.

In studying the effect of p_H on the activity of the enzyme, the dialysed enzyme free from ammonium sulphate was used, and glucose was added at its optimum concentration. No buffer was added, since the protein in the solution possesses a large buffering power. It was found that at p_H 6–8, the activity at 37° remained approximately constant. At p_H 9–9·5 the activity was apparently greater, but this was found to be due, at least in part, to the increased reducing power towards methylene blue of the enzyme solution alone. In still more alkaline solutions, glucose by itself slowly reduces methylene blue. In phosphate buffer of p_H 8·0, glucose by itself showed no reduction of methylene blue even after 10 hours at 37°.

The glucose dehydrogenase is completely precipitated, together with all the pigments, by saturation with ammonium sulphate. Attempts to precipitate the enzyme without precipitating the pigments were not very successful, as the enzyme is only partially precipitated even by 75% saturation with ammonium sulphate. Owing to the difficulty of obtaining the enzyme free from ammonium

sulphate, a number of other possible precipitants were tried without success. The precipitates obtained with 50%, 75%, and 90% acetone showed little or no activity even on carrying out the precipitation with ice-cold acetone. Similarly, the product obtained by precipitation with 90% alcohol keeping the mixture below 0° was inactive.

The enzyme may be filtered without loss through kieselguhr, whereby some pigment is removed and freedom from insoluble cell material is ensured. The enzyme may also be filtered through a fine porcelain candle (Pasteur-Chamberland L 5) without loss of activity. With the concentrated enzyme, filtration may be slow unless the solution is first filtered through kieselguhr. The dilute solution obtained after dialysing and centrifuging the extract of acetone-liver, however, filters through the candle readily and can then be saturated with ammonium sulphate yielding a preparation free from cell debris and giving a perfectly clear solution in water.

A number of attempts were made to purify and concentrate the enzyme by adsorption methods. Most of these experiments were carried out with the dialysate from acetone-sheep liver before precipitation with ammonium sulphate. It was found difficult to standardise the conditions since different enzyme preparations contained different amounts of protein. Treatment of 20 cc. of dialysate (p_H 5·7) five times with 0·5 g. of charcoal (Merck's Medicinal), stirring for a minute and filtering under suction each time, followed by precipitation with ammonium sulphate usually gave an enzyme containing much less pigment and with its activity unimpaired. By continuing the charcoal treatment, more pigment is removed, but the enzyme is also adsorbed from the solution. The enzyme does not appear to be adsorbed from the untreated dialysate by kaolin or kieselguhr either in acid or alkaline solution. If, however, part of the protein be first removed by treating about five times with charcoal, the enzyme can be adsorbed from acid solution by kaolin. Thus, by stirring 10 cc. of the charcoal-treated dialysate three times with 0·5 g. kaolin at p_H 4·5 or 5·5, about two-thirds of the enzyme was removed from the solution. Treatment of the kaolin with $N/10$ K_2HPO_4 followed by precipitation of the eluted enzyme with ammonium sulphate gave a solution with some activity towards glucose, though much of the enzyme was lost.

The effect of temperature on the destruction of the enzyme depends on the nature of the enzyme preparation. For example, the presence of ammonium sulphate appears to protect the enzyme. A solution of the ammonium sulphate-precipitated enzyme, after heating at 52° in nitrogen at p_H 7·3 for 5 minutes and centrifuging off the precipitate of inactive protein, was found to have lost little of its activity. On the other hand, the enzyme dialysate before precipitation with ammonium sulphate, warmed under the same conditions, lost nearly half of its activity. The ammonium sulphate-precipitated enzyme solution at p_H 7·3 lost about one-third of its activity after heating at 45° for half an hour. The enzyme is rapidly destroyed at 60° even in presence of ammonium sulphate.

The reduction of methylene blue by glucose in presence of the enzyme was not inhibited by the presence of neutralised $M/300$ KCN. The addition of 0·048 mg. (= 1 unit) of insulin hydrochloride (British Drug Houses) in a total volume of 2·1 cc. produced no effect on the rate of reduction of methylene blue. The enzyme is not destroyed by toluene, and several preparations were made by grinding liver with toluene, extracting with water and precipitating the enzyme with ammonium sulphate.

The aerobic oxidation of glucose.

A large number of experiments were carried out in the Barcroft apparatus to determine whether the system glucose-oxidising enzyme *plus* glucose was capable of taking up oxygen directly. The results have been rather inconclusive, for while with a considerable number of preparations of the enzyme, the oxygen uptake has been somewhat greater in presence of glucose than in its absence, this extra uptake being unaffected by KCN, yet with many other preparations of the enzyme, no oxygen uptake with glucose has been obtained. Most of these experiments were carried out with the ammonium sulphate-containing preparation, but in those experiments in which the dialysed ammonium sulphate-free enzyme was used, it is noteworthy that in no case was an oxygen uptake observed. It was also noted that, with most enzyme preparations, at the end of the experiment the solution containing glucose was definitely more acid than at the beginning, and more acid than the control without glucose. These observations suggested the following explanation as possibly accounting for the apparent oxygen uptake observed in some cases. The glucose, while not being directly oxidised by oxygen, may possibly be able to use as a hydrogen acceptor some reducible substance in the enzyme preparation, for example, a reducible pigment. The formation of an acid oxidation product of glucose oxidised in this manner would account for the development of acidity in the solution. (Formation of lactic acid by glycolysis has already been excluded.) At the beginning of all the experiments, the solution was definitely alkaline (p_H 7·4 or 7·6). When the ammonium sulphate-containing preparation was used, free ammonia would thus be present in the gas phase. During the course of the experiment, the development of acidity in the solution would cause the absorption of this ammonia gas, which would be read on the manometer as an apparent uptake of oxygen.

When methylene blue was added to the solution in the Barcroft apparatus, an oxygen uptake with glucose was always observed, the methylene blue being alternately reduced by glucose and oxidised by molecular oxygen. It seemed therefore that in the oxidation of glucose with glucose dehydrogenase, molecular oxygen cannot act as a hydrogen acceptor. It was thus of interest to see whether the aerobic oxidation could be brought about in presence of active oxygen.

Oxidation of glucose in presence of the cytochrome and indophenol oxidase system.

Keilin [1929] has shown that cytochrome is oxidised by oxygen activated by indophenol oxidase and that it is reduced by dehydrogenase systems. Cytochrome is believed to act as a carrier between the oxygen activators and the hydrogen activators of the cell. Keilin [1930] has shown that the oxidation of cysteine is greatly accelerated by indophenol oxidase in presence of cytochrome, the latter acting as a carrier between the activated oxygen and the sulphydryl group. The oxidation of an actual dehydrogenase system by cytochrome *plus* indophenol oxidase has not hitherto been experimentally brought about. One of the difficulties is that the indophenol oxidase which is prepared from heart muscle, itself contains certain dehydrogenases. Preliminary experiments with methylene blue having shown that the indophenol oxidase prepared from heart muscle does not contain any glucose dehydrogenase, an attempt was made to build up the complete oxidising system using glucose dehydrogenase, that is, to find whether glucose *plus* glucose dehydrogenase would take up oxygen in presence of cytochrome *plus* indophenol oxidase. The following experiments and the curves in Fig. 2 show that in presence of the complete system there is a rapid oxygen uptake.

A fresh solution of cytochrome *c* was prepared from 2 lbs. of Delft yeast by the first method of Keilin [1930]. The precipitate obtained with SO_2 was well washed, suspended in water, brought to p_H 7·4 with NaOH and made up to 7 cc. Indophenol oxidase was prepared by a method similar to that of Keilin by grinding fresh sheep's heart muscle with fine washed sand, using 50 cc. of dry sand to 20 g. of muscle. The very fine suspension in water was centrifuged, washed twice with distilled water and evenly suspended in phosphate buffer, 14 g. of wet pulp being made up to a volume of 50 cc. and brought to p_H 7·4. This very strong suspension was used, since during the experiment the particles become aggregated on the sides of the vessel, the enzyme surface being thereby much reduced. The glucose dehydrogenase solution was an ammonium sulphate-free preparation prepared as already described. At 37°, 0·4 cc. (equivalent to 1 g. of dry liver) + 0·6 cc. p_H 7·4 buffer reduced 0·5 cc. methylene blue in 10 minutes in presence of 0·025 cc. 2*M* glucose and in 30 minutes without glucose. Four Barcroft differential manometers were used, the right-hand bottle of each receiving 1·7 cc. of glucose dehydrogenase. The first received in addition 0·8 cc. p_H 7·4 phosphate buffer; the second, 0·8 cc. buffer and 0·2 cc. 2*M* glucose; the third, 0·8 cc. indophenol oxidase and 0·3 cc. cytochrome; the fourth, 0·8 cc. oxidase and 0·3 cc. cytochrome and 0·2 cc. 2*M* glucose. Water was added to make the volume in each bottle up to 3·0 cc. The left-hand bottles each contained 3·0 cc. buffer. The side-tube of all bottles contained 5% NaOH soaked on a roll of filter-paper to absorb CO_2. The apparatus were shaken in a water-bath at 37°.

The oxygen uptakes after 110 minutes were as follows:

	mm.³	Final p_H
1. Dehydrogenase	83	7·2
2. Dehydrogenase + glucose	85	7·2
3. Dehydrogenase + cytochrome + oxidase	189	7·0
4. Dehydrogenase + cytochrome + oxidase + glucose	352	6·7

The course of the oxygen uptake is shown in the corresponding curves in Fig. 2.

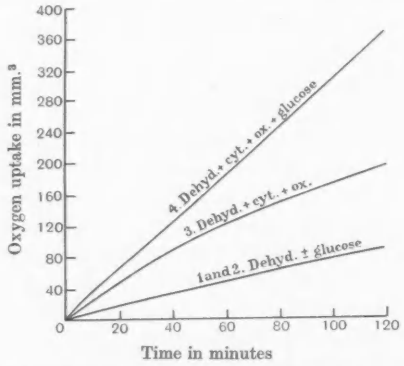

Fig. 2. Oxygen uptake of glucose in presence of dehydrogenase, cytochrome and indophenol oxidase.

It is clear that while no oxidation of glucose was brought about by the dehydrogenase itself, a large oxidation of glucose was induced by the dehydrogenase in presence of the oxygen-activating system, cytochrome and indophenol oxidase. Incidentally, the greater uptake in 3 compared with 1 shows that the cytochrome-oxidase system also increased the oxygen uptake of the residual reducing systems in the dehydrogenase preparation.

This experiment has been repeated a number of times with different preparations of the enzymes, and similar results have always been obtained. In spite of the highly buffered solutions, the p_H at the end of the experiment was always definitely lower in the solution in which oxidation of glucose had taken place. It appears that the oxidation product of glucose is an acid.

The following experiment was carried out to show that the complete system, glucose dehydrogenase, cytochrome and indophenol oxidase, is necessary in order to bring about the oxidation of glucose; *i.e.* that neither cytochrome nor the oxidase alone will induce the oxidation. The cytochrome, oxidase and dehydrogenase solutions were the same as used in the previous experiment, stored overnight at 0°. Five Barcroft apparatus were used, the solutions in the two bottles of any one apparatus being the same except that each right-hand bottle contained glucose while each left-hand bottle contained an equal volume of water. The bottles of No. 1 each contained 1·7 cc. glucose dehydrogenase and 0·8 cc. p_H 7·4 phosphate buffer; No. 2 contained 1·7 cc. dehydro-

genase and 0·8 cc. indophenol oxidase; No. 3 contained 1·7 cc. dehydrogenase, 0·3 cc. cytochrome and 0·8 cc. buffer; No. 4 contained 1·7 cc. dehydrogenase, 0·3 cc. cytochrome and 0·8 cc. oxidase; No. 5 contained 0·3 cc. cytochrome, 0·8 cc. oxidase but no dehydrogenase. After adding 0·2 cc. 2*M* glucose to each of the right-hand bottles, sufficient water was added to make the volume in each bottle equal to 3·0 cc.

The oxygen uptake after 170 minutes at 37° was as follows:

	mm.³
1. Dehydrogenase + glucose	19
2. Dehydrogenase + glucose + oxidase	0
3. Dehydrogenase + glucose + cytochrome	7
4. Dehydrogenase + glucose + cytochrome + oxidase	101
5. Glucose + cytochrome + oxidase	18

It is clear that the only solution showing an appreciable oxygen uptake is that containing the complete system, glucose with its dehydrogenase, cytochrome and indophenol oxidase. (In some experiments, an oxidation of glucose has been induced by indophenol oxidase without added cytochrome. Keilin has found, however, that the heart-muscle indophenol oxidase always contains a small amount of cytochrome. The oxidation of glucose in presence of oxidase *plus* added cytochrome has always been much greater.)

In order to exclude the possibility that the results in this experiment might be due to glycolysis of glucose to lactic acid by the indophenol oxidase preparation, followed by oxidation of the lactic acid, the following experiment was carried out. The manometric experiment was performed exactly as before except that the undialysed dehydrogenase preparation was used and the temperature was 30°. At the same time, to each of four small conical flasks were added 3·5 cc. dehydrogenase and 1·65 cc. indophenol oxidase. 0·41 cc. 2*M* glucose + 0·62 cc. water were added to two flasks *A* and *A'*, while duplicate control flasks *B* and *B'* received 1·03 cc. water but no glucose. (The concentrations are the same as those used in the manometric experiments.) The flasks were quickly filled with nitrogen, stoppered and incubated for 3½ hours at 30°. 14 cc. of water and 6 cc. 20 % trichloroacetic acid were then added to each flask, and after removal of glucose by the copper-lime method, lactic acid was estimated by the method of Friedemann, Cotonio and Shaffer [1927]. The results were as follows:

	Lactic acid mg.
A with glucose	0·47
A' with glucose	0·47
B without glucose	0·42
B' without glucose	0·47

It is evident that no measurable quantity of lactic acid has been formed from the glucose. The manometric experiment which was carried out simultaneously at 30°, gave similar results to that described above, namely no appreciable oxygen uptake except where the complete system, glucose with its dehydrogenase, cytochrome and indophenol oxidase, was present. This shows

therefore that the oxygen uptake is due to the direct oxidation of glucose rather than to the oxidation of lactic acid formed by glycolysis of the glucose.

Discussion.

This appears to be the first time that a soluble glucose dehydrogenase has been isolated from the cell. The only reference in the literature to the extraction of an enzyme which oxidises glucose appears to be that of Müller [1929] who obtained a glucose oxidase from *Aspergillus niger*. This enzyme is entirely different in its properties from the enzyme which I have described. Müller's enzyme does not bring about the oxidation of glucose in presence of methylene blue and does in presence of oxygen. Like tyrosinase, therefore, it is an oxidase and not a dehydrogenase. The resistance of glucose dehydrogenase to toluene suggests that it is different from the glucose-oxidising enzyme which is present in suspensions of certain bacteria; Quastel and Wooldridge [1927] have found that in "resting" *B. coli* the enzyme which brings about the oxidation of glucose is completely inactivated by treatment with toluene.

I have not been able to find any example in the literature of an oxidising enzyme which can be filtered through kieselguhr and a fine porcelain candle without loss of activity. The lactic dehydrogenase prepared by Stephenson [1928] from *B. coli* could be filtered through kieselguhr but was completely inactivated by passing the solution through a porcelain candle. The observation that glucose dehydrogenase may be filtered through a fine porcelain candle is of interest as indicating that the activity of the enzyme is associated with molecules in true colloidal solution. The molecules constituting the enzyme or to which the enzyme is attached are doubtless large and extremely complex, but the enzyme activity is evidently not to be ascribed to a suspension of microscopic remnants of the broken cell. It has been shown that glucose dehydrogenase with glucose does not take up oxygen to any appreciable extent. Also, the indophenol oxidase preparation from washed heart muscle does not contain any glucose dehydrogenase. These two facts rendered the glucose dehydrogenase a particularly suitable enzyme for testing the ability of cytochrome *plus* indophenol oxidase to induce the aerobic oxidation of a substrate activated by an anaerobic dehydrogenase. The experiments described above, showing that in presence of glucose dehydrogenase, cytochrome and indophenol oxidase, glucose is oxidised by oxygen, afford the first direct demonstration of the co-operation of the oxidase and cytochrome with a dehydrogenase in bringing about the aerobic oxidation of the substrate of the last of these.

It is premature to speculate on the function in the body of glucose dehydrogenase until more is known about the distribution of the enzyme and until the oxidation product of glucose has been identified. The acid formed by the oxidation of glucose is now being investigated.

SUMMARY.

1. An enzyme (glucose dehydrogenase) has been obtained by saturating the water extract of acetone-liver with ammonium sulphate. The enzyme has been extracted from the liver of the ox, sheep, cat and dog.

2. The enzyme is obtainable in a perfectly clear, cell-free solution which can be filtered through a porcelain candle or through kieselguhr.

3. The enzyme brings about the oxidation of glucose in presence of methylene blue as hydrogen acceptor.

4. This anaerobic oxidation of glucose is not inhibited by KCN, NaF, toluene or iodoacetic acid.

5. Glucose is not oxidised in the presence of oxygen, an oxygen activator apparently being necessary.

6. The system glucose and glucose dehydrogenase has been combined with Keilin's cytochrome-indophenol oxidase system in presence of which the aerobic oxidation of glucose readily occurs, cytochrome acting as a carrier between active oxygen from the oxidase and active hydrogen from glucose *plus* its dehydrogenase.

7. The oxidation product of glucose has not yet been determined, but it appears to be an acid. (See Note below.)

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Note added July 24th, 1931.

It has now been found that the oxidation product of glucose is gluconic acid. This has been isolated as the characteristic calcium salt, from which the pure phenylhydrazide has been prepared. The latter melted at 199°, the melting-point being unchanged by mixing with an authentic specimen. The cinchonine salt of the acid melted at 186° after recrystallisation from 95% alcohol. Gluconic acid appears to be the main, if not the sole, product of the oxidation. It is hoped shortly to publish these results in detail.

CXIV. SYNTHESIS OF THE *d*- AND *l*-3:4-DIHYDROXYPHENYLALANINES.

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Although *dl*-3 : 4-dihydroxyphenylalanine has been synthesised on several occasions [Funk, 1911; Stephen and Weizmann, 1914; Harington and McCartney, 1927], the optically active forms of this amino-acid have not so far been artificially obtained. *l*-3 : 4-Dihydroxyphenylalanine was first isolated by Guggenheim [1913] from *Vicia faba*; renewed biological interest attaches to this amino-acid as the result of the recent work of Raper [1926], who has shown that it represents the first stage in the formation of melanin from tyrosine by the enzyme tyrosinase. It therefore appeared to us desirable to complete the synthesis of *l*-3 : 4-dihydroxyphenylalanine by resolution of the *dl*-amino-acid into its optically active components.

We attempted first to formylate the *dl*-amino-acid with the intention of subsequently resolving the formyl derivative by means of suitable alkaloidal salts. The formyl derivative turned out, however, to be an excessively soluble substance which was also difficult to handle owing to the extreme ease with which it was oxidised.

We then turned our attention to the preparation of a triacetyl derivative of dihydroxyphenylalanine which would be free from the objection of ready oxidisability. Having failed, in this case, to obtain any good result by the elegant method of acetylation with ketene, recently described by Bergmann [1930], we proceeded as follows. Protocatechuic aldehyde was condensed with acetylglycine in presence of acetic anhydride and sodium acetate. The azlactone formed in this reaction is much less stable than is generally the case, and on standing in contact with water is converted into β-3 : 4-diacetoxyphenyl-α-acetaminoacrylic acid. Reduction of this compound to the corresponding propionic acid derivative by means of sodium amalgam in the ordinary way was excluded by the ease with which the acetoxy-groups were hydrolysed in alkaline solution. It could, however, be effected readily and in good yield by a catalytic method. The resulting β-3 : 4-diacetoxyphenyl-α-acetaminopropionic acid was treated with one equivalent of hydrated brucine in concentrated alcoholic solution. Crystallisation set in very slowly and was not complete until a fortnight had elapsed. During the crystallisation a smell of ethyl acetate

developed and it was found in fact that the salt which had separated was the brucine salt of *l*-*β*: 3: 4-dihydroxyphenyl-*α*-acetaminopropionic acid, elimination of the O-acetyl groups having occurred during the protracted crystallisation. The salt of the corresponding *d*-acid was obtained from the mother-liquor.

The free acids, as was to be expected, were excessively soluble compounds and very readily oxidisable. They could not be isolated satisfactorily and the solutions recovered from the salts after removal of the alkaloid in the usual way were, therefore, submitted directly to hydrolysis with hydrochloric acid. In this way there were obtained preparations of the *d*- and *l*-amino-acids having respectively $[\alpha]_D$ + 11·9° and − 12·0°. The *l*-amino-acid corresponded in all its properties with a sample of the natural acid isolated from *Vicia faba*.

Experimental.

β-3 : 4-Diacetoxyphenyl-α-acetaminoacrylic acid. Protocatechuic aldehyde (21·6 g.), acetylglycine (18·4 g.) and freshly fused sodium acetate (12·8 g.) were ground together to form an intimate mixture. This was treated with freshly distilled acetic anhydride (60 cc.) and the whole was heated on the steam-bath for 2 hours. After cooling, the dark-coloured viscous solution was poured into cold water (750 cc.). The sticky yellow gum which separated hardened on keeping overnight. It was collected, washed with water and crystallised from ethyl acetate. A further crop was obtained by concentrating the ethyl acetate mother-liquor, stirring up the residue with water, keeping overnight and then proceeding as before. The total yield was 18 g. For analysis the acid was recrystallised from water, decoloration being effected with charcoal. It separated in glistening white plates. The air-dried material sintered at 115°–120° and melted at 183·4°. On heating at 100° it lost $1H_2O$ and the anhydrous product melted at 187–8°.

(Found for the anhydrous product: C, 56·1; H, 4·8; N, 4·4 %. $C_{15}H_{15}O_7N$ requires: C, 56·1; H, 4·7; N, 4·4 %. 300 mg. of the air-dried material lost 17·3 mg. in 3 hours at 100°; whence loss of weight = 5·8 %; calculated for $1H_2O$ 5·3 %.)

β-3 : 4-Diacetoxyphenyl-α-acetaminopropionic acid. The above-described unsaturated acid (28 g.) was dissolved in warm water (4000 cc.) The solution was cooled to the ordinary temperature, and, after addition of 1 g. of palladium chloride (dissolved in water with the aid of the minimum amount of hydrochloric acid), was shaken in an atmosphere of hydrogen. The uptake of hydrogen ceased after 2 hours, by which time 2600 cc. had been absorbed. On keeping the solution overnight the catalyst flocculated and could easily be removed by filtration. The filtrate was digested with a suspension of well-washed silver carbonate; after removal of the precipitate the solution was freed from excess of silver with hydrogen sulphide and concentrated under diminished pressure to about 800 cc. when separation of crystalline material commenced. The crystals were collected after the solution had stood overnight and amounted to 17·4 g.; a further crop of 3 g. was obtained by concentrating the mother-liquor to about 75 cc.

The acid was recrystallised from water from which it separated in small colourless blunt prisms, M.P. 171–172°. It is readily soluble in hot water, sparingly so in cold. It does not absorb bromine nor does it decolorise alkaline permanganate immediately.

(Found: C, 55·6; H, 5·3; N, 4·4 %. $C_{15}H_{17}O_7N$ requires: C, 55·7; H, 5·3; N, 4·3 %.)

Brucine l-β-3 : 4-dihydroxyphenyl-α-acetaminopropionate. dl-β-3 : 4-Diacetoxyphenyl-α-acetaminopropionic acid (27 g.) and brucine (38·5 g. of the hydrated alkaloid) were dissolved together in absolute alcohol (270 cc.) and the solution was set aside. Crystallisation commenced in about 24 hours. After 14 days the solid, which appeared not to be increasing in amount any further, was filtered off, washed with a little alcohol and dried. The yield was 19 g. The salt was recrystallised from 95 % alcohol from which it separated in colourless needles containing $2H_2O$; attempts to remove the water of crystallisation failed owing to decomposition of the salt at the high temperature required; on heating in a capillary tube it sintered at 157°, and melted with effervescence at 176°.

It was readily soluble in water, sparingly so in absolute alcohol, and insoluble in other organic solvents.

After two recrystallisations the rotation became constant

$$\alpha^{20°}_{5461} = -1{\cdot}89° \ (c = 2{\cdot}04, \ l = 2);$$

whence

$$[\alpha]^{20°}_{5461} = -46{\cdot}3°.$$

The strong smell of ethyl acetate which had developed during the slow separation of this salt had suggested the possibility of de-acetylation. That such de-acetylation had indeed occurred was shown by the analysis of the salt, the figures agreeing well with those required by the brucine salt of *N*-acetyl-3 : 4-dihydroxyphenylalanine with $2H_2O$. (Found: C, 61·48; H, 6·47; N, 6·64 %. $C_{34}H_{39}O_9N_3, 2H_2O$ requires: C, 61·1; H, 6·43; N, 6·28 %.) Further, the salt gave with ferric chloride in neutral aqueous solution an intense green coloration which passed into reddish-purple on addition of sodium bicarbonate, indicating that the adjacent hydroxyl groups attached to the benzene ring were free.

Brucine d-β-3 : 4-dihydroxyphenyl-α-acetaminopropionate. The alcoholic mother-liquor after removal of the crude insoluble brucine salt was concentrated under diminished pressure to a syrup. This was kept for a week, during which time it partly solidified. The pasty mass was boiled out three times in succession with ethyl acetate and then triturated with ether until it became wholly granular. The crude dried material (24 g.) was crystallised from water, in which it was very soluble at the boiling point but sparingly so in the cold. After two recrystallisations the optical rotation became constant

$$\alpha^{20°}_{5461} = +0{\cdot}31° \ (c = 2{\cdot}04, \ l = 2);$$

whence

$$[\alpha]^{20°}_{5461} = +7{\cdot}6°.$$

The salt formed long narrow prisms containing $2H_2O$, which sintered at 150° and melted with effervescence at 154°.

(Found: C, 61·46; H, 6·51; N, 6·35 %. $C_{34}H_{39}O_9N_3$, $2H_2O$ requires: C, 61·1; H, 6·43; N, 6·28 %.)

d-3 : 4-Dihydroxyphenylalanine. The laevorotatory brucine salt described above was dissolved in water and the solution, cooled in ice, was treated with excess of saturated aqueous barium hydroxide. The mixture was stirred vigorously with a glass rod for about 5 minutes whilst still in the ice in order to accelerate the separation of the alkaloid. This was filtered off rapidly and the last traces were removed by extraction with chloroform, the air in the separating-funnel being displaced with hydrogen. With as little delay as possible the aqueous solution was treated with just sufficient dilute sulphuric acid to precipitate all the barium, brought to the boil and filtered. The filtrate was concentrated under diminished pressure to about 15 cc. for every 5 g. of salt taken, treated with strong hydrochloric acid to make a final concentration of 10 % HCl and boiled under reflux for 1 hour. The solution was then evaporated to dryness under diminished pressure; the residue was dissolved in a little water, and ammonia was cautiously added, under a layer of light petroleum, until present in slight excess. The whole was then rapidly evaporated to dryness under diminished pressure in a stream of hydrogen. The crystalline residue was purified by dissolving in boiling water containing a trace of sulphur dioxide and treatment with charcoal; the filtered solution deposited stout colourless prisms of the amino-acid on cooling. The amino-acid melted at 282° with decomposition. In 5·01 % solution in *N* hydrochloric acid the observed rotations were $\alpha_{5461} + 1{\cdot}33°$ and $\alpha_D + 1{\cdot}20°$ respectively in a 2 dm. tube; whence $[\alpha]_{5461} = + 13{\cdot}2°$ and $[\alpha]_D = + 11{\cdot}9°$.

l-3 : 4-Dihydroxyphenylalanine. This was obtained in a precisely similar manner from the alcohol-soluble dextrorotatory brucine salt and formed colourless prisms, M.P. 282° (decomp.). In 5·04 % solution in *N* hydrochloric acid the observed rotations in a 2 dm. tube were $\alpha_{5461} - 1{\cdot}30°$ and $\alpha_D - 1{\cdot}21°$ respectively, whence $[\alpha]_{5461} = - 12{\cdot}9°$ and $[\alpha]_D = - 12{\cdot}0°$. A sample of natural *l*-3 : 4-dihydroxyphenylalanine, kindly supplied by Dr M. Guggenheim, had M.P. 281° (decomp.); this M.P. was unaltered by admixture with the synthetic material. The natural acid had $[\alpha]_{5461} - 13{\cdot}6°$ and $[\alpha]_D - 12{\cdot}0°$ ($c = 5{\cdot}3$, and $l = 2$). The identity of the two acids is, therefore, established.

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CXV. THE ISOLATION OF *d*-3:5-DIIODOTYROSINE FROM THE THYROID GLAND BY THE ACTION OF PROTEOLYTIC ENZYMES.

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About two years ago the present authors [1929] described the isolation of *dl*-3 : 5-diiodotyrosine by means of graduated hydrolysis of the thyroid gland with barium hydroxide. The isolation was repeated shortly afterwards by Foster [1929] who used a simpler method and obtained a better yield. Although in the previous work every precaution was taken to exclude the possibility of artificial production of diiodotyrosine during the course of the isolation, and although such artificial production was in any case very unlikely to occur, it remained nevertheless desirable to complete the demonstration of the natural occurrence of diiodotyrosine in the thyroid by its isolation by gentler means and in the optically active condition.

Promising material for this investigation offered itself in the various mother-liquors containing acid-soluble organically combined iodine which were obtained as by-products in the course of the work of Harington and Salter [1930] on the isolation of *l*-thyroxine by the enzymic digestion of thyroid gland.

As a first step the experience gained in previous work on diiodotyrosine was utilised to effect a preliminary concentration of the iodine-containing compounds present in the above-mentioned mother-liquors. Thus the mother-liquor obtained after removal of the material insoluble at p_H 5·0, following on the peptic digestion of crude iodothyreoglobulin, contained much iodine, a considerable proportion being present as iodide but a definite amount being still in organic combination. After preliminary clearing of this solution with lead acetate, practically the whole of the iodine was precipitated with silver nitrate in neutral or faintly alkaline solution. The organic iodine compounds were separated from iodide by extraction of the precipitated silver salts with dilute nitric acid and re-precipitated by addition of ammonia; decomposition of the re-precipitated silver salts in the usual manner yielded a solution of the organic iodine compounds in high concentration which was subjected to further digestion with trypsin.

After the tryptic digestion was complete the solution was subjected to a

process of fractionation closely similar to that employed in the previous work [Harington and Randall, 1929] on the isolation of diiodotyrosine, yielding finally a soluble gummy material containing much iodine and giving an intense reaction for diiodotyrosine with nitrous acid and ammonia, but at the same time still giving a biuret reaction.

A product with similar characteristics was obtained by analogous concentration of the organic iodine-containing compounds which remained in solution after removal of the thyroxine-containing fraction of the tryptic digestion products of iodothyreoglobulin [*cf.* Harington and Salter, 1930].

The final successful isolation of diiodotyrosine was achieved by subjecting the combined solution of a number of such end-products of tryptic digestion to the action of an active preparation of erepsin. After this treatment it was possible by a simple fractionation to separate diiodotyrosine in the pure condition.

The losses involved at the various stages of the procedure outlined above were very heavy and the amount of diiodotyrosine finally obtained has no quantitative significance. Evidence was offered previously [Harington and Randall, 1929] bearing on the probable amount of diiodotyrosine actually present in the thyroid, and the suggestions made at that time have since been largely confirmed by the improved yield obtained by Foster [1929]. The present work is to be regarded as the final evidence that *d*-diiodotyrosine does actually occur as a constituent amino-acid of the characteristic protein of the thyroid.

Experimental.

Isolation of d-*3 : 5-diiodotyrosine from the acid-soluble fraction of peptic digestion products from iodothyreoglobulin.*

The filtrate from a peptic digestion of crude iodothyreoglobulin [*cf.* Harington and Salter, 1930], amounting to 7200 cc. and containing in all 1·986 g. of iodine, was treated with a concentrated aqueous solution of 350 g. of lead acetate and then with sodium hydroxide until just neutral to litmus. The solution was filtered, and the filtrate, containing 1·4 g. of total iodine, was made faintly alkaline to litmus with ammonia and treated with silver nitrate to complete precipitation. The insoluble silver salts were filtered off, suspended in about 800 cc. of water, and treated with freshly boiled out nitric acid until the suspension was permanently acid to Congo red. The silver halides were removed as rapidly as possible by filtration, and the organic silver salts were re-precipitated from the filtrate by the cautious addition of ammonia until present in slight excess. The precipitate was collected, thoroughly washed with water, suspended in 1000 cc. of water and decomposed with hydrogen sulphide. The filtrate from the silver sulphide, containing 423 mg. of iodine was concentrated under diminished pressure to 360 cc.

This solution, which was slightly acid, was brought to p_H 8·0 by addition of sodium hydroxide and was treated with 0·8 g. of trypsin (Merck) dissolved in

10 cc. of water. Toluene was added as a preservative and the solution was placed in an incubator at 38°. At the commencement of the digestion the total N amounted to 20·0 mg./cc. and the amino-N to 1·0 mg./cc. After 48 hours the amino-N had increased to 2·65 mg./cc.; a further addition of 0·8 g. of trypsin was made at this point, which, in the course of another 24 hours, increased the amino-N to 3·01 mg./cc. At this stage the digestion was interrupted; the solution was diluted to 2 litres, neutralised and brought to the boil. A saturated solution of uranium acetate was then added until no further precipitation occurred. The precipitate was removed and the excess of uranium was thrown out with ammonia. The filtered solution was concentrated to 200 cc. and contained 271 mg. of iodine. Silver nitrate was added to complete precipitation, and the insoluble silver salts were fractionated by extraction with nitric acid as described above.

The solution obtained by recovery from the organic silver salts contained 174 mg. of iodine. It was concentrated to 200 cc. and extracted by shaking out 9 times at 70° with purified (aldehyde-free) butyl alcohol. The butyl alcohol extracts were evaporated to dryness under diminished pressure, the evaporation being several times repeated after addition of water. The residue contained 128 mg. of iodine; it was dissolved in water (600 cc.) and the clearing process with uranium acetate was repeated. After removal of the uranium the solution, containing 109 mg. of iodine, was concentrated under diminished pressure to 140 cc. and precipitated with basic lead acetate. After standing overnight the precipitate was filtered off, suspended in water and decomposed with hydrogen sulphide. The filtrate from the lead sulphide, when concentrated, yielded a yellow gum which contained 67 mg. of iodine all in organic combination. The material gave a strong colour reaction for diiodotyrosine with nitrous acid and ammonia, but gave also a biuret reaction and could not be induced to crystallise.

The residues from two such experiments, containing in all 142 mg. of iodine, were therefore united in solution in 30 cc. of water; 60 cc. of an active erepsin extract were added and the mixture was brought to p_H 8·0, saturated with toluene and placed in an incubator at 38°. At the commencement of the digestion the mixture contained total N 2·18 mg./cc. and amino-N 0·51 mg./cc.; the digestion was interrupted after 67 hours when the amino-N had reached 0·96 mg./cc. and the rate of increase had become very low.

The solution was diluted with 50 cc. of water and treated with excess of silver nitrate. The insoluble silver salts were filtered off, washed and decomposed with hydrogen sulphide. The filtrate from the silver sulphide (containing 124 mg. of iodine) was freed from hydrogen sulphide and cleared with uranium acetate; after removal of excess of uranium the solution was concentrated to 80 cc. and contained 100 mg. of iodine. Basic lead acetate was added in slight excess, and after standing overnight the precipitate was collected, washed, suspended in water and decomposed with hydrogen sulphide. The lead sulphide was thoroughly boiled out several times with water to extract adsorbed diiodotyrosine. The filtrate and extracts from the lead sulphide, which con-

tained 88 mg. of iodine, were concentrated to a low bulk under diminished pressure.

On keeping overnight an amorphous precipitate, admixed with nodules of colourless needles, had separated. The latter were collected and the mother-liquor was cleared on the centrifuge and concentrated further, when a second crop of crystals was obtained. A still further amount of crystals was obtained by dissolving the amorphous precipitate in boiling 50 % acetic acid and allowing the solution to cool. The whole of the crystalline material was collected and recrystallised from 50 % acetic acid, yielding 77 mg. of prismatic needles. (Found: N, 3·18 %; calc.: N, 3·2 %.) The compound gave the characteristic colour reaction with nitrous acid and ammonia with intensity; it had M.P. 194° (decomp., uncorr.) and this M.P. was unchanged by admixture with a sample of *d*-3 : 5-diiodotyrosine prepared by the iodination of *l*-tyrosine. In 3·64 % solution in *N* hydrochloric acid, using a 0·5 dm. tube, the observed rotations of the material from the thyroid were $\alpha_{5461} + 0{\cdot}07°$ and $\alpha_D + 0{\cdot}05°$; whence $[\alpha]_{5461} + 3{\cdot}85°$ and $[\alpha]_D + 2{\cdot}75°$. Abderhalden and Guggenheim [1908] record $[\alpha]_D + 3{\cdot}0°$ for the acid dissolved in 4 % hydrochloric acid.

Isolation of d-*3 : 5-diiodotyrosine from the acid-soluble fraction of tryptic digestion products of iodothyreoglobulin.*

The filtrates obtained by removal of all material insoluble at p_H 5·0 after the tryptic digestion of iodothyreoglobulin [*cf.* Harington and Salter, 1930] were subjected to a process of fractionation similar to that just described for the tryptic digest of the acid-soluble peptic products. The procedure in one such instance is summarised below.

	Iodine (mg.)
Tryptic filtrate (3600 cc.)	481
After clearing with uranium acetate	348
After precipitating with silver nitrate, fractionation of silver salts and recovery	168
After butyl alcohol extraction	153
After second uranium treatment	119

The gummy residues from four such experiments, containing in all 318 mg. of iodine, were united; one-half was subjected to digestion with erepsin at p_H 8·0 for 93 hours. The digest was worked up as before and yielded finally 150 mg. of crude crystalline *d*-3 : 5-diiodotyrosine.

Note on preparation of active erepsin extract.

The following method has been found convenient for the preparation of active erepsin extract.

The mucous membrane of the small intestine of a recently killed dog was scraped off with a spatula and ground up with sand in a mortar under 5 times its weight of 66 % glycerol. After standing overnight the mixture was diluted with 4 vols. of water and rapidly centrifuged. The extract was submitted to dialysis under diminished pressure in an apparatus of the type described by Sørensen [1918] until free from glycerol and was finally concentrated by

filtration through the collodion bag until the volume was one-half that of the original extract. A slight precipitate was removed by filtration through ordinary filter-paper. The resulting solution was kept at 0° in presence of toluene, but was used as soon as possible. It contained a very active erepsin, being capable of hydrolysing glycylglycine quantitatively in a few hours.

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CXVI. THE METABOLISM OF IODOBENZENE AND *p*-IODOPHENYLMERCAPTURIC ACID IN THE RAT AND THE RABBIT.

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The present work was undertaken as a preliminary step in the investigation of the experimental haemoglobinuria described by Callow and Hele [1927]. These workers found that *p*-bromo- and *p*-chloro-phenylmercapturic acids, whether given by the mouth or subcutaneously, cause a transient haemoglobinuria in the dog. An attempt was made to find a small animal which would show a similar response to dosage with mercapturic acids. This attempt has not so far been successful; but in the course of the work certain interesting features of the sulphur metabolism of rats and rabbits were displayed. The possibility of mercapturic acid formation from iodobenzene and the effect of dosage with *p*-iodophenylmercapturic acid were studied in both these animals.

The ability of the rabbit to synthesise *p*-bromophenylmercapturic acid has been shown by Abderhalden and Wertheimer [1925], and some evidence in favour of the occurrence of this synthesis in the rabbit has been brought forward by Rhode [1922], and by Shiple, Muldoon and Sherwin [1924] in the case of the pig. The evidence suggesting a synthesis of mercapturic acid by the rabbit, brought forward by Rhode, was very inconclusive, being founded solely upon the diminished synthesis of ethereal sulphate from monohalogen benzene derivatives when cystine was given simultaneously.

Evidence in favour of the oxidation of mercapturic acid to ethereal sulphate has been adduced by Shiple, Muldoon and Sherwin [1924] for the case of the pig, and by Rose, Shiple and Sherwin [1924] for the case of the rabbit. On the other hand, previous work in this laboratory by Coombs and Hele [1927] indicated that this oxidation does not occur in the pig, and a criticism of the conclusions drawn by Sherwin and his co-workers is given in their paper.

No case of haemoglobinuria following the administration of a mercapturic acid has been described, other than the observations of Callow and Hele quoted above; nor has haemoglobinuria been obtained, either in the rat or the rabbit, in the course of the present work.

Experimental.

I. *Experiments upon the rat.*

An inbred, piebald strain was used. The degree of toxicity of iodobenzene and *p*-iodophenylmercapturic acid for the rat is indicated by the

following data. It was found that iodobenzene could be given to adult male rats, by subcutaneous injection, in doses of 1·5 mg. per g. of body weight, every three days, for 12–18 days, before any marked deterioration in the animal's general condition occurred. A single dose of this size was lethal within 48 hours when given intraperitoneally. *p*-Iodophenylmercapturic acid was given as the aqueous solution of its sodium salt, and a dose of 1 mg. per g. of body weight was lethal in over 50 % of cases. A dose of 0·6 mg. per g. of body weight was well tolerated when given subcutaneously. Mr Innes, of the Department of Animal Pathology, Cambridge, has kindly submitted the kidneys, livers and intestines of animals used in the above experiments to histological examination, and found no gross pathological changes.

In all experiments upon the rat, the following synthetic diet, made into a paste with distilled water, was fed *ad lib.*

"Light white casein"	23 %
Starch	40 %
Cane sugar	17 %
Salt mixture	5 %
Palm kernel oil	15 %

Cod-liver oil and marmite were added to the above diet in the proportions of 2·5 % and 7·5 % respectively. In each experiment two Hopkins metabolism cages were set up, each cage containing two adult male rats. Each morning, the urine which had been passed during the preceding 24 hours, from all four rats, was mixed with the rinsings from the collecting apparatus, made up to a suitable volume with distilled water and filtered before analysis.

Analytical Methods.

Total sulphur, total sulphate and inorganic sulphate were determined by the methods used in this laboratory for the analysis of dog's urine [Hele, 1924]. The ethereal sulphates and neutral sulphur were obtained by difference. Iodine was estimated by the method of Kendall [1920] with the exception that one incineration only was required in order to destroy completely the organic matter present in 10 cc. of urine. Iodine estimated in alkaline solutions of *p*-iodophenylmercapturic acid was found in approximately the proportion of 92 % of that present.

Exp. 1. In this experiment iodobenzene was twice given subcutaneously. The results of the sulphur analyses are recorded graphically in Fig. 1. The dosage, and a summary of the resultant changes in the sulphur partition are given in Table I A. Owing to the small quantity of urine available for analysis no satisfactory estimations of neutral sulphur and ethereal sulphate were obtained for the 4th day of this experiment.

Exp. 2. *p*-Iodophenylmercapturic acid was given once mixed with the food, when about one-third of its iodine was detected in the urine; and once by stomach tube. The results of the sulphur analyses are recorded graphically in

Fig. 2. The dosage, and a summary of the resultant changes in the sulphur partition are given in Table I B.

Table I.

Experiment and dose	Period in days	Neutral S daily av. mg. S	Ethereal SO_4 daily av. mg. S	Total extra neutral S mg. S	Total extra ethereal SO_4 mg. S	% dose excreted as sulphur compound	% dose excreted as iodine compound
A. 4 ♂ Rats. Figures calculated per rat							
Average figures for 9 normal days	1–6, 10–13	6·8	0·9	—	—	—	—
On 7th day 0·18 g. C_6H_5I subcutaneously	7–9	—	—	12·4	3·5	56	102
On 14th day 0·34 g. C_6H_5I subcutaneously	14–19	—	—	38·9	4·0	80	102
B. 4 ♂ Rats. Figures calculated per rat							
Average figures for 12 normal days	1–8, 13–16	7·2	1·0	—	—	—	—
On 9th day 0·75 g. *p*-iodophenylmercapturic acid mixed with food	9–12	—	—	20·5	nil	43	33
On 17th day 0·20 g. ditto by stomach tube	17–18	—	—	12·5	nil	71	32
C. Rabbit I. ♀							
Average figures for 28 normal days	1–10, 13–18, 25–32, 35–38	180*	13	—	—	—	—
On 11th day 5·4 g. C_6H_5I by stomach tube	11–12	—	—	nil*	280	33	54
On 19th day ditto subcutaneously	19–24	—	—	92*	182	32*	98
On 33rd day 3·0 g. and on 34th day 2·0 g. *p*-iodophenylmercapturic acid by stomach tube	33–34	—	—	234*	nil	53*	64
D. Rabbit II. ♂							
Average figures for 12 normal days	1–12	8·2	3·0	—	—	—	—
On 13th and 14th days 2 g. *p*-iodophenylmercapturic acid by stomach tube	13–14	—	—	312	nil	89	73

* The significance of these figures is uncertain, owing to the irregularity of the neutral sulphur excretion.

Exp. 3. Isolation of p*-iodophenylmercapturic acid from rat urine.* Sixteen adult male rats, caged in Hopkins metabolism cages, were used in this

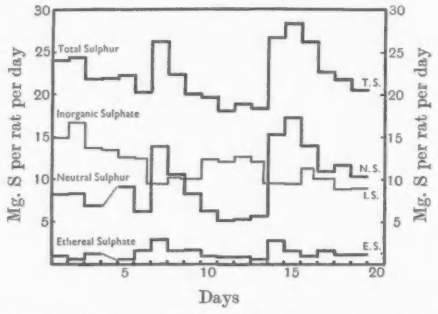

Fig. 1. Dosage. On 7th day, 0·18 g. iodobenzene per rat, subcutaneously.
„ 14th „ 0·34 „ „ „

experiment. Urine was collected daily, and stored over chloroform. The full synthetic diet described above was fed throughout. Each rat received 0·36 g. of iodobenzene subcutaneously every three days, until a total of 34·6 g. had been given. Five litres of urine were obtained, and worked up in two batches of $2\frac{1}{2}$ litres each.

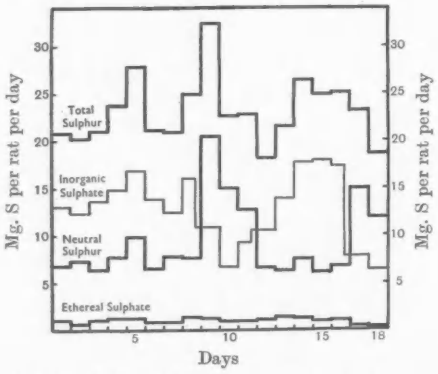

Fig. 2. Dosage. On 9th day, 0·75 g. *p*-iodophenylmercapturic acid per rat, mixed with food.
„ 17th „ 0·20 „ „ „ given by stomach tube.

Treatment of first batch. This batch was separated into excess chloroform, precipitate, and filtrate. Analysis showed that nearly all the iodine was in the filtrate. An attempt was made to isolate *p*-iodophenylmercapturic acid from this fraction by Baumann's method, which had given good results with dog's urine in this laboratory. This method, however, failed to remove coloured impurities, and only a very small yield of pigmented material melting at 151° and decomposing at 187° was obtained. Attempts to decolorise this material

showed that the mercapturic acid was strongly adsorbed on to Merck's medicinal charcoal, and this property was utilised in the treatment of the second batch.

Treatment of second batch. The urine was filtered, and 600 cc. of the filtrate used in preliminary trials of the method. The remainder was acidified with hydrochloric acid and allowed to stand for eight days. The precipitate obtained was dissolved in dilute ammonia, and the solution shaken with Merck's medicinal charcoal until the filtrate gave no precipitate on acidification. The charcoal was then dried at 37° and extracted with successive 100 cc. portions of 97 % alcohol, until on evaporation of the alcohol only traces of solid residue were obtained. Approximately 20 g. of charcoal and 2 litres of alcohol were required.

The preliminary precipitation with hydrochloric acid and solution of this precipitate in ammonia removed 1·6 g. of the total iodine present in batch 2 of urine. Of this, 1·39 g. was absorbed on the charcoal. The extraction with alcohol removed 1·06 g. from the charcoal, and the final purified product contained 0·61 g. of iodine, thus giving a yield of mercapturic acid of 8·2 % from the iodobenzene injected.

Analysis of this product gave the following figures:

	N	S	I
Found	3·84	9·06	35·4
Theoretical for *p*-iodophenylmercapturic acid	3·84	8·77	34·8

The melting-point (152°) was unchanged on mixing with various proportions of pure *p*-iodophenylmercapturic acid, prepared in this laboratory from the urine of dogs which had been fed with iodobenzene.

II. *Experiments upon the rabbit.*

The rabbit was found to tolerate iodobenzene very readily. A dose as high as 5 g. given by subcutaneous injection to a rabbit weighing 3 kg. produced only a transient diminution of appetite. A similar slight reaction was observed in the case of the doe rabbit used in Exp. 4, after giving, by stomach tube, a dose of *p*-iodophenylmercapturic acid of 1·9 mg. per g. body weight. This rabbit was fed upon a diet of oats and cabbage. The buck rabbit used in Exp. 5, and fed upon a diet of sugar-beet, died 24 hours after receiving, by stomach tube, the second instalment of a dose of *p*-iodophenylmercapturic acid of 2·1 mg. per g. body weight.

The diet used in Exp. 4 consisted of 150 g. minced cabbage and 20 g. Sussex oats, given morning and evening. In Exp. 5, however, this diet was changed, in order to decrease the output of urinary sulphur. In this experiment the rabbit received 50 g. of sliced sugar-beet morning and evening.

In both experiments, the rabbit was kept in a cage with a wire floor, set in a large zinc funnel. Beneath the outlet of this funnel was placed a Hopkins separator of rather larger size than that used in rat work. Efficient separation

of urine and faeces was thus obtained. On every alternate morning the collecting apparatus was rinsed with distilled water, and the combined rinsings and urine were made up to a suitable volume, acidified with acetic acid to dissolve the copious phosphatic precipitate usually present, and filtered before analysis. In the case of the buck used in Exp. 5, catheterisation was carried out at the end of each two-day period of collection of urine. This procedure was not practicable in the case of the doe used in Exp. 4.

The same analytical methods were used as in the experiments upon the rat. Nitrogen was estimated by the Kjeldahl method.

Exp. 4. An adult doe rabbit, weighing approximately 2·5 kg. was used in this experiment. Iodobenzene was given once by stomach tube and once by subcutaneous injection. *p*-Iodophenylmercapturic acid was given once by stomach tube. The results of the sulphur analyses are shown graphically in Fig. 3. The dosage, and a summary of the resultant changes in the sulphur partition are given in Table I C.

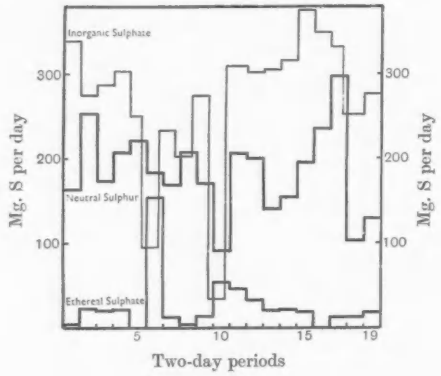

Fig. 3. Rabbit 1.

Dosage. On 1st day of 6th period, 5·4 g. iodobenzene by stomach tube.
" " 10th " " " subcutaneously.
" " 17th " 3·0 g. *p*-iodophenylmercapturic acid by stomach tube.
" 2nd " 17th " 2·0 " " " "

Exp. 5. An adult buck rabbit, weighing approximately 2·0 kg. was used in this experiment. *p*-Iodophenylmercapturic acid was given by stomach tube.

Particulars of this experiment are given in Table I D.

Exp. 6. In order to determine whether the rabbit was capable of synthesising a mercapturic acid from iodobenzene, to an extent too small to be detected by estimations of the neutral sulphur, the following experiment was carried out.

As a preliminary test, a rabbit was injected subcutaneously with 3·6 g. of iodobenzene. The urine passed during the next six days was treated as in the case of the second batch of rat urine in Exp. 3. About 50 mg. of somewhat impure *p*-iodophenylmercapturic acid were obtained, having M.P. 148°–149°, and giving the following analytical figures: N, 3·64; S, 9·06; I, 34·8.

Six adult doe rabbits were then placed upon a diet of carrot and oats, fed *ad lib.*, and housed in metabolism cages. Each rabbit received 3·6 g. iodobenzene subcutaneously and five days later a further injection of 3·6 g. The urine was collected daily for ten days after the first injection and preserved over chloroform. This urine was rendered distinctly alkaline to litmus with ammonia, and the phosphate precipitate filtered off. Its volume was then 5600 cc., and it contained about 10 g. of iodine. Treatment of this material according to the method described in Exp. 3 yielded 0·64 g. of impure *p*-iodophenylmercapturic acid, having M.P. 149°–150°. After purification by recrystallisation of the ammonium salt, the melting-point was 150°, and analysis gave the following figures: N, 3·70; S, 8·88; I, 34·75.

Discussion of Results.

I. *Experiments upon the rat.* Fig. 1 shows that the chief change brought about in the sulphur partition by a dose of iodobenzene is an increase in the neutral sulphur and total sulphur fractions. The ethereal sulphate shows only a slight increase. A large increase in the excretion of neutral sulphur, such as that occurring after the second dose of iodobenzene, may have been due, in part at least, to toxic effects of the dose. This possibility is shown more strikingly in the experiments with mercapturic acid (Fig. 2). Dosage with *p*-iodophenylmercapturic acid leads to an increase in the neutral sulphur excretion greater than that which would correspond to the mercapturic acid excreted, as judged by the iodine analyses. This excess of neutral sulphur appeared to be directly related to the severity of the signs of poisoning shown by the rats. In no case did dosage with *p*-iodophenylmercapturic acid cause haematuria or haemoglobinuria in the rat. The indication of mercapturic acid synthesis given by Exp. 1 was followed up in Exp. 3 by isolation of *p*-iodophenylmercapturic acid from rat urine. Thus the metabolism of iodobenzene in the rat is similar to its metabolism in the dog; but the rat differs from the dog in that it shows no haemoglobinuria after dosage with mercapturic acid.

II. *Experiments upon the rabbit.* Administration of iodobenzene to the rabbit, either orally or subcutaneously, causes a large increase in the ethereal sulphate output and a corresponding drop in the inorganic sulphate. No indication of mercapturic acid formation is given by the neutral sulphur figures (Fig. 3), although the conversion of a very small fraction of injected iodobenzene into *p*-iodophenylmercapturic acid is conclusively demonstrated in Exp. 6. The large fall in the excretion of all sulphur fractions, except ethereal sulphate, on the 10th day of Exp. 4 is accounted for by the fact that the animal refused its food for one day after this injection.

The effect of giving *p*-iodophenylmercapturic acid to rabbits is shown in Figs. 3 and Table I C and D. Both experiments show clearly that no appreciable oxidation of this substance to ethereal sulphate has occurred. This result contrasts with the findings of Sherwin and his co-workers, who stated that the rabbit oxidises *p*-bromophenylmercapturic acid to ethereal sulphate to the

extent of 23 % to 30 % [Rose, Shiple and Sherwin, 1924]; and that the pig is capable of oxidising phenyl-, *p*-chlorophenyl- and *p*-bromophenyl-mercapturic acids to ethereal sulphate to the extent of 38 %, 43 % and 23 % respectively. Previous work in this laboratory [Coombs and Hele, 1927], however, indicated that neither *p*-bromophenyl- nor *p*-iodophenyl-mercapturic acid could be oxidised to ethereal sulphate by the pig. The rise in the neutral sulphur excretion after giving *p*-iodophenylmercapturic acid is not well shown in Fig. 3 (Exp. 4) on account of the large and irregular quantities of neutral sulphur excreted throughout this experiment. In Exp. 5, a diet was chosen which gave a very low sulphur excretion, and the rise in neutral sulphur is very evident. In this experiment the rise in neutral sulphur is in excess of that which would correspond with the iodine excretion. This condition is doubtless due to the severe poisoning which occurred in the case of this animal. No haematuria or haemoglobinuria was observed in these experiments upon the rabbit.

Summary.

(1) It has been shown that the rat is able to synthesise *p*-iodophenylmercapturic acid from iodobenzene; is unable to oxidise that mercapturic acid to ethereal sulphate; and does not develop haemoglobinuria after the administration of that mercapturic acid.

(2) The rabbit is unable to oxidise *p*-iodophenylmercapturic acid to ethereal sulphate. It does not develop haemoglobinuria after the administration of that mercapturic acid. It is able to convert a very small fraction of injected iodobenzene into *p*-iodophenylmercapturic acid.

I wish to express my gratitude to Dr T. S. Hele for his interest and advice in this work. The expenses of this research were in part defrayed by a grant to Dr Hele from the Government Grant Committee of the Royal Society.

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CXVII. OBSERVATIONS ON SOME ALCOHOL-SOLUBLE PROTEINS FROM MILK PRODUCTS.

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The presence of alcohol-soluble proteins in milk or cheese has been reported by several workers. Weidemann [1882] isolated from Emmenthal cheese by extraction with boiling ethyl alcohol a substance which he called caseoglutin. This was confirmed by Rose and Schulze [1884] and Benecke and Schulze [1887]. Winterstein and Thöni [1902], Winterstein [1904] and Bisegger [1907], also working with Emmenthal cheese, concluded that caseoglutin is formed during the ripening process. Nierenstein [1912], working with old Cheddar cheese, isolated from 6874 g. of cheese 82 g. of dried caseoglutin containing 14·52 % N. Osborne and Wakeman [1918] isolated from caseinogen prepared from milk a protein which was soluble in 50–70 % alcohol and very dilute acetic acid and gave a voluminous precipitate with potassium ferrocyanide. The tryptophan, Millon and biuret reactions were strongly positive. The phosphorus content was much less than that of caseinogen, and less arginine, histidine and lysine were found on hydrolysis. Anaphylactic reactions showed no genetic relationship between caseinogen and this alcohol-soluble protein. Grimmer and Wagenführ [1925] and Grimmer and Schutzler [1926] concluded from the analysis of the extract from Backstein cheese with 70 % alcohol that the caseoglutin obtained was a mixture of various substances, and that the composition was very variable.

Experimental.

In the course of the extraction of amino-acids from Cheddar cheese by the butyl alcohol method [Dakin, 1918] the presence of an alcohol-soluble protein was discovered.

To effect a preliminary separation of the amino-acids and lower polypeptides from caseinogen and the higher protein decomposition products in cheese 80 % alcohol was used. Powdered fat-free dried cheese was extracted with this reagent for 24 hours and the filtrate was evaporated at 40° under reduced pressure to obtain an aqueous residue for further extraction with butyl alcohol. It was found, however, that a bulky white precipitate was

formed as the ethyl alcohol was removed. The precipitate failed to dissolve on the addition of water to the aqueous residue but when ethyl alcohol was added until the concentration reached 80 % a clear solution was again obtained. A preliminary examination of the substance showed that it was a protein with a phosphorus content much less than that of caseinogen. A detailed investigation of its nature was therefore undertaken.

(*a*) *Protein from immature cheese.* An 18 weeks old Cheddar cheese was thoroughly grated and extracted with ether for 5 hours. The residue was dried in air for 24 hours at 55° to a dry, friable powder. 100 g. were suspended in 1 litre of 80 % alcohol and shaken at intervals over a period of 24 hours. The mixture was filtered and all the alcohol removed under reduced pressure at 40°. The bulky white precipitate, which was glutinous in parts, was filtered off and washed with cold water. On taking up in 80 % alcohol a slightly turbid solution was obtained. The turbidity was probably due to the denaturation of a portion of the protein since it had been previously noticed that the flocculent portion dissolved completely. On distilling this alcoholic solution at 40° under reduced pressure, filtering, washing the precipitate with water, and redissolving it in 80 % alcohol the amount of denaturation was found to be increased, with a lowering of the solubility.

The protein was precipitated from alcoholic solution with 2 volumes of ether, filtered and washed well with ether. This process was repeated once and the solid dried in air. In this way 0·8 g. of a pure white, friable powder was obtained.

Properties of the protein. The protein had the following properties.

(1) It was soluble in dilute alkali and re-precipitated from the solution on the gradual addition of dilute acid.

(2) An aqueous suspension dissolved on the addition of a little acetic acid.

(3) It was insoluble in water and absolute alcohol but completely soluble in 80 % alcohol. The colloidal suspension formed by adding water to this solution was flocculated by the addition of hydrochloric acid and could then be filtered.

(4) Sodium acetate gave no precipitate when added to the alcoholic solution. A fine white precipitate was produced by ether.

(5) Potassium ferrocyanide produced a bulky white precipitate from a solution in dilute acetic acid.

(6) The biuret, xanthoproteic and Millon reactions were positive and the Adamkiewicz and the Reichl reactions for tryptophan were also given.

(*b*) *Protein from mature cheese.* Investigations were made in order to see whether the same or a similar protein could be recovered from mature cheese. For this purpose a Cheddar cheese 16 months old, made from the same milk at the same time as the immature cheese, was used. 200 g. of fat-free, air-dried cheese were suspended for 24 hours in 1 litre of 80 % alcohol and then filtered. The filtrate was added to 3 volumes of water and the resulting precipitate separated by filtration. This light yellow precipitate failed to redissolve in

cold 80 % alcohol and became very glutinous. On warming to 40° some solution occurred, but separation into two layers took place on cooling, a slightly yellow, hard precipitate adhering to the bottom of the flask and a white, flocculent precipitate forming the top layer.

The white, flocculent precipitate was decanted and filtered, yielding a white, friable residue. Redissolving this residue in warm 80 % alcohol and adding the solution to two volumes of cold water produced a milky, colloidal suspension. On adding dilute alkali drop by drop a point was reached where the protein separated into flakes which filtered easily, leaving a clear filtrate of p_H 4·5. After drying in air at 30° the residue yielded 3·28 g. of a white, friable powder. The reactions were those of the protein isolated from the immature cheese.

The glutinous nature of the protein when mixed with alcohol appeared to be similar to that of the monocalcium paracaseinate of Van Slyke [1924] but its negligible ash content and insolubility in warm 5 % sodium chloride solution discounted this idea.

(c) *Protein from peptic digest of caseinogen.* In view of the presence in cheese of alcohol-soluble proteins an examination of the peptic digest of caseinogen for similar compounds was made.

100 g. of B.D.H. "Light White Casein" were extracted several times with 80 % alcohol at p_H 5·0, to remove any alcohol-soluble protein present, washed in ether and dried in air. Digestion with pepsin in 500 cc. of water acidified with hydrochloric acid was then carried out for 104 hours in the presence of chloroform. The volume was made up to 2½ litres with absolute alcohol, the mixture well shaken and the p_H adjusted to 5·0. Extraction was continued with occasional shaking for 15 hours and the solution filtered. When the alcohol was removed by distillation *in vacuo* at 40° a bulky precipitate separated. This was filtered, washed in ether, taken up three times in warm 80 % alcohol and finally dried. A yield of 0·25 g. of a white powder was obtained. The reactions were those of the first two proteins.

Composition of the three alcohol-soluble proteins. After drying *in vacuo* over sulphuric acid micro-combustions were carried out in duplicate on each protein to determine the carbon, hydrogen, nitrogen, sulphur, ash and moisture contents, by Dr Schoeller of Berlin. Table I shows the results of analysis.

Table I. *Composition of three alcohol-soluble proteins.*

Protein from ...	Cheese 4 months old	Cheese 16 months old	Peptic digest of caseinogen
C	52·43	52·04	52·51
H	7·98	7·55	7·26
N	14·36	14·15	14·38
S	0·93	0·83	1·02
Ash	0·003	Trace	0·004
Moisture	5·26	6·95	3·83

Phosphorus estimations. Colorimetric estimations by Briggs's [1922] method indicated a very low phosphorus content in all cases but by the method of

Benedict and Theis [1924], which avoids loss of phosphorus during incineration, appreciably higher values were obtained. For accurate determinations the method of Fiske and Subbarow [Hawk, 1927] and a dry ashing process were used. The method was tried out on a sample of caseinogen (prepared by the method of Van Slyke [1924]). Duplicate estimations gave values of 0·742 and 0·751 % for the phosphorus content of the moisture-free caseinogen. The values obtained for the phosphorus content of the three proteins were as follows:

Protein from ...	Cheese 4 months old	Cheese 16 months old	Peptic digest of caseinogen
P %	0·19	0·08	0·03

Discussion.

The composition of the three alcohol-soluble proteins, computed on a moisture- and ash-free basis, is shown in Table II, where the composition of caseinogen is also given for comparison.

Table II. *Composition of alcohol-soluble proteins and of caseinogen.*

Protein	Caseinogen	Cheese 4 months old	Cheese 16 months old	Peptic digest of caseinogen
C	53·11	55·34	55·93	54·60
H	7·05	7·68	7·23	7·03
N	15·65	15·16	15·21	14·95
S	0·82	0·98	0·89	1·06
P	0·82	0·20	0·09	0·03
O	22·55	20·64	20·65	22·33

It is evident that the proteins differ considerably in composition from caseinogen and show some appreciable differences between themselves. Their main characteristic is their very low phosphorus content. Reference to Table I shows that the ash content of each is negligible, so that it may be assumed that the phosphorus present is in organic combination and is not an inorganic contamination.

Although the three proteins exhibit very similar characteristics and each has a very low phosphorus content they are not identical in composition. It is therefore important to decide whether the proteins from the cheese were initially present in the milk from which the cheese was made or whether they are decomposition products of the casein. The protein isolated by Osborne and Wakeman [1918] from caseinogen prepared from milk is very similar to the three proteins discussed in this paper and has a low phosphorus content—0·08 %. However, the caseinogen used by these authors was prepared from milk by precipitation with dilute hydrochloric acid, the product being purified by dissolving in alkali and precipitating with acid several times. If, therefore, we regard the alcohol-soluble protein finally isolated from the caseinogen as being initially present in the milk, it must be assumed that such treatment is without effect on the caseinogen molecule.

Rimington and Kay [1926] found that during the action of pepsin on

caseinogen no measurable amount of free phosphoric acid was liberated. Trypsin, however, converted rather more than 50 % of the phosphorus into free phosphoric acid in 2 months, while 1 % alkali at 37° liberated all the phosphorus as phosphoric acid within 24 hours. Rimington [1927] isolated from a tryptic digest of caseinogen a phosphopeptone which contained 50 % of the organic phosphorus. The molecule of this compound contained nine amino-acid linkages and was split off from caseinogen with great rapidity. Posternak [1927] isolated three similar compounds from a tryptic digest of caseinogen, but Rimington suggested that the presence of a phosphorus-free compound which followed the precipitation of the phosphopeptone rather closely was responsible for the variety of compounds obtained by this author.

Several workers have recently claimed that caseinogen itself is not homogeneous but is a mixture of several different proteins. Kondo [1923–25] and Linderstrøm-Lang [1925–27] were led to this conclusion by the fact that the solubility of caseinogen in dilute hydrochloric acid was found to be a function of the amount of undissolved caseinogen and that by fractional precipitation fractions could be obtained which differed considerably in P/N ratios. Linderstrøm-Lang [1927–29], using 60 % alcohol as a differential slovent, obtained from caseinogen several fractions with very different phosphorus contents. By re-combining the fractions it was possible to secure a mixture which exhibited the same properties (*e.g.* specific rotation, solubility in 60 % alcohol, solubility in hydrochloric acid, rate of rennet coagulation) as the original caseinogen.

Svedberg and co-workers [1930], by the application of the sedimentation velocity method and the equilibrium method [Svedberg, 1926, 1927], found that caseinogen prepared by Hammarsten's method consisted of several proteins of different molecular weights. By extracting caseinogen with 70 % alcohol acidified with hydrochloric acid they obtained a protein which they found to be a definite chemical entity, with a molecular weight of 375,000. Caseinogen prepared by the method of Van Slyke [1924] was also found to be heterogeneous.

There is thus some evidence for the conclusion that caseinogen in the free condition is a mixture of several different proteins. In any method of preparation, however, the use of dilute acid and alkali is essential for the purposes of preparation and purification and, having regard to the fact that caseinogen has been shown to be easily acted upon by alkali and by trypsin, resulting in the removal of the phosphorus, it appears very probable that the caseinogen molecule is so delicately constituted and the linkages so fragile that the treatment with acid and alkali to which it is subjected in the course of preparation is sufficient to rupture the molecule in several places, giving rise to a mixture of proteoses of lower molecular weight and differing phosphorus contents.

On these grounds, therefore, it is considered that the two proteins isolated from cheese are probably mixtures of several similar compounds, resulting

from breakdown of the casein either by the rennin, bacterial enzymes, or possibly the action of acid. The attempt to obtain a similar alcohol-soluble protein from the peptic digestion of caseinogen resulted, it is true, in a yield of only 0·25 g. from 100 g. of caseinogen. However, the caseinogen used was first extracted several times with 80 % alcohol and it is possible that this treatment removed nearly all the alcohol-soluble protein which was capable of being formed from the caseinogen and that treatment of the remainder with pepsin resulted mainly in the formation of other and lower degradation products.

SUMMARY.

Three alcohol-soluble proteins—two from Cheddar cheese and one from a peptic digest of caseinogen—have been isolated and their properties studied. The proteins differ considerably from caseinogen in composition and have a very low phosphorus content. Evidence is cited for the view that they are decomposition products of the caseinogen or the casein.

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CXVIII. THE FERRICYANIDE METHOD FOR THE DETERMINATION OF REDUCING SUGARS. A MODIFICATION OF THE HAGEDORN-JENSEN-HANES TECHNIQUE.

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The modification of the Hagedorn and Jensen [1923] method for the estimation of reducing sugars introduced by Hanes [1929] has the advantage over copper reduction methods that much smaller amounts of sugar can be accurately estimated. Also, as Hanes showed, this method, unlike the copper reduction methods, gives results that are not appreciably affected by the oxygen tension in the solution being estimated. He employed the method for the estimation of glucose and maltose, the latter sugar giving a linear relation between the amount of sugar and amount of ferricyanide reduced while glucose did not give such a linear relation under Hanes's conditions.

Callow [1930] applied the Hanes method to the estimation of invert sugar, but the method has not been standardised for fructose.

In order to use this method for a study of carbohydrate metabolism in green leaves, employing small quantities of material, it was first necessary to investigate the sugar/ferricyanide relation for fructose and for varying mixtures of the reducing sugars (glucose, fructose and maltose). It was hoped also that it might be possible to find a set of conditions under which the sugar/ferricyanide relationship would be linear for all the reducing sugars. Under such conditions one might expect that the total reducing power would be the simple sum of the reducing values of the component sugars. This would enormously simplify the analysis of the extracts. From the results of Hanes and Callow it seemed likely that this might be attained by altering the time of boiling, or the volume or both. As the results of this paper show, this possibility has been realised by changing the volume alone.

Experimental.

Hanes's technique for the actual oxidation of the sugar by ferricyanide and the titration of the excess of ferricyanide with thiosulphate was carefully followed with the exception that the volume of sugar solution taken was 10 cc. in place of 5 cc.

The thiosulphate solution (approximately $N/75$) was standardised against standard potassium iodate by the method given by Hanes. It was found advisable to check the strength of this thiosulphate solution every 2 or 3 days, especially in warm weather. The chemicals used were of the A.R. quality as supplied by the British Drug Houses, Ltd.; the proportions used were exactly as given by Hanes.

Sugar samples.

Glucose. The glucose used was Dextrose A.R. (British Drug Houses), which had been dried *in vacuo* over phosphorus pentoxide to constant weight. The sugar so treated had an optical rotation of $[\alpha]_D^{20^\circ} + 52{\cdot}78^\circ$ in an 8 % solution; the calculated rotation was $[\alpha]_D^{20} + 52{\cdot}68^\circ$. In order to check the dryness of the sample the true weight of glucose in a solution made by dissolving 5 g. of the above sample in distilled water and then making to 100 cc. was ascertained by making use of the density tables of Brown, Morris and Millar [1897]. By this method it was found that exactly 5 g. of glucose were present in this solution.

Invert sugar. This sugar was prepared from Saccharose A.R. (B.D.H.) which was ground to a fine powder and dried under diminished pressure over phosphorus pentoxide to constant weight, by the method of Lane and Eynon [1923]. A 10 % unneutralised solution had $[\alpha]_D^{20^\circ} - 20{\cdot}62^\circ$; the calculated value, allowing for the acid present, was $[\alpha]_D^{20^\circ} - 20{\cdot}72^\circ$. The rotation of the sucrose from which the invert sugar was prepared was $[\alpha]_D^{20^\circ} + 66{\cdot}39^\circ$ in a 2 % solution; calculated value $[\alpha]_D^{20^\circ} + 66{\cdot}42^\circ$.

Fructose. The fructose used was a sample of Kahlbaum's "Fructose kryst" which was ground to a fine powder and dried under diminished pressure at 60° over phosphorus pentoxide to constant weight. It had $[\alpha]_D^{27^\circ} - 86{\cdot}40^\circ$. This rotation is within the range of published values for fructose but is rather low compared with the theoretical value calculated from the formula suggested by Vosburgh [1920]. This fact tended to show that, although dried to constant weight under the conditions described, the fructose was not completely dry. This was proved by the fact that a solution containing 5 g. of the sample in 100 cc. of solution was found, from density determinations (as for glucose above), to contain actually only 4·930 g. of dry fructose. Hence apparent weights of fructose must be multiplied by 4·93/5·00 to give the true weight of dry fructose. This correction is embodied in all the results given for fructose; its application leads to the following optical rotation for the fructose sample used: $[\alpha]_D^{27^\circ} - 87{\cdot}68^\circ$, the theoretical value being $- 88{\cdot}08^\circ$.

Maltose. The sample used was a Kahlbaum preparation of pure maltose monohydrate which had been recrystallised three times from 60 % alcohol and dried *in vacuo* as above to constant weight. The $[\alpha]_D^{20^\circ}$ of an 8 % solution was $+ 130{\cdot}02^\circ$; calculated value $+ 130{\cdot}07^\circ$ [Parcus and Tollens—see Landolt, 1898].

The calculated values for the optical rotations of the sugars were obtained from the formulae given by Landolt [1898] unless otherwise stated.

Sugar solutions.

Two different methods of delivering the required amounts of sugar in solution were employed.

(*a*) Sugar solutions of 0·01 and 0·05 % were prepared and transferred to the boiling-tubes containing the ferricyanide from a 10 cc. burette (graduated to 1/50 cc. and fitted with a fine waxed jet) in the required quantities.

(*b*) Sugar solutions of 0·01, 0·02, 0·25 and 0·05 % were prepared and transferred to the boiling-tubes by means of accurate 2, 5 and 10 cc. pipettes.

In both cases the final volumes of sugar solution were made to 10 cc. with distilled water using a graduated pipette, when necessary. Water volume differences of 0·01 cc. had no measurable effect on the results.

Both methods (*a*) and (*b*) were used indiscriminately although in most cases duplicate estimations using the two methods were carried out, good agreement being obtained. In all cases the total number of duplicates was four and the maximum variation in cc. thiosulphate ($N/75$ approx.) among replicates was 0·03 cc.

Standardisation data.

Table I gives the mean values obtained for glucose, invert sugar, fructose and maltose. The actual readings of approximately $N/75$ thiosulphate have been converted to their $N/100$ equivalents for the sake of uniformity.

Table I.

Sugar mg.	cc. $N/100$ thiosulphate $-(WB-R)\times$ normality factor (mean of four estimations)			
	Glucose	Invert sugar	Fructose	Maltose
0·20	—	—	0·54	0·38
0·25	0·69	0·69	0·67	—
0·50	1·44	1·43	1·43	1·05
0·75	2·17	2·16	2·12	—
1·00	2·90	2·89	2·88	2·18
1·25	—	—	—	2·70
1·50	4·32	4·36	4·36	3·24
1·75	—	—	—	3·79
2·00	5·83	5·87	5·82	4·34
2·50	7·31	7·36	7·30	5·42
3·00	8·76	8·82	8·72	6·545
3·50	—	—	—	7·66

Above 3·0 mg. in the case of glucose, fructose and invert sugar and above 3·5 mg. in the case of maltose the results are not sufficiently accurate. This is due, no doubt, to the large percentage of the total ferricyanide used in the oxidation of the sugar in these cases.

The sugar-thiosulphate reaction.

From inspection of the glucose results it is clear that equal increments in sugar give approximately equal increments in thiosulphate, 0·25 mg. glucose corresponding on the average to 0·735 cc. thiosulphate. The observed value for an initial 0·25 mg. glucose is, however, 0·69 cc. so that although the

relation is approximately linear the line will not pass through the origin. The relationship between sugar and thiosulphate may therefore be put in the form

$$S = b\,(T + a),$$

a representing an amount in cc. thiosulphate to be added to the observed thiosulphate value, T, and b the conversion factor for thiosulphate to sugar, S. A similar relation holds for all the sugars under investigation. In future thiosulphate value T will be distinguished from the value $(T + a)$ by designating the former "observed thiosulphate value" and the latter "reducing value," T and a being expressed in terms of $N/100$ thiosulphate unless otherwise stated.

Fig. 1 shows the experimentally determined points for glucose and maltose (two separate series of determinations) and the lines corresponding to the calibration equation finally adopted, *i.e.* for glucose $S = 0{\cdot}340\ (T + 0{\cdot}05)$—see later.

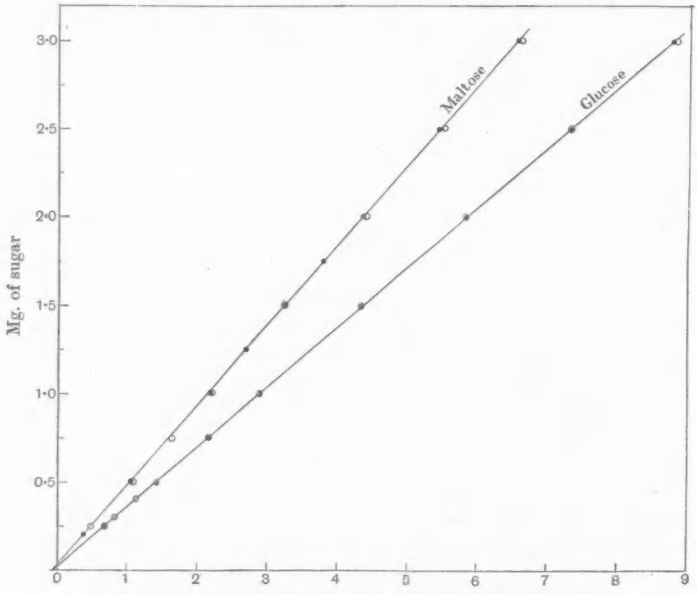


Fig. 1.

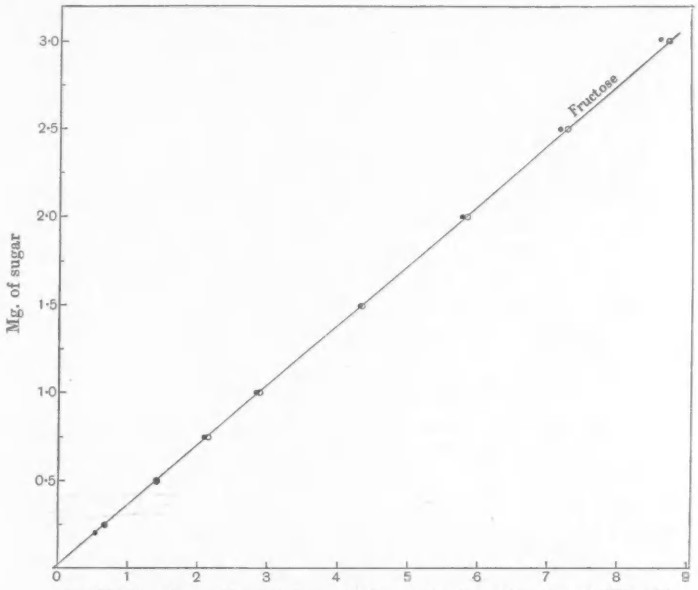
Values of a and b were obtained by the method of least squares taking the calculations to the third and fourth decimal place respectively. The values obtained are given in Table II.

As a measure of the accuracy with which these values fit the observations

Table II.

Sugar	a	b	S.D.$_c$
Glucose	0·041	0·3409	0·0065
Fructose	0·052	0·3410	0·0063
Invert sugar	0·059	0·3377	0·0046
Maltose	0·053	0·4545	0·0063

we give also, as S.D.$_c$, the standard deviation of calculated from observed values in mg. sugar.



Cc. $N/100$ sodium thiosulphate equivalent to ferricyanide reduced ($WB - R$)

Fig. 2.

Consideration of factors a and b.

As it was impossible to read the thiosulphate burette, which was graduated in 0·02 cc. divisions, to a greater accuracy than 0·01 cc. the factors a and b can only have significance to the second and third decimal place respectively. Further, it is clear that the values of a are not significantly different for the different sugars and the mean value 0·05 can be used without loss of accuracy. Using this value of a and taking b to the third decimal place, the following rationalised values were obtained (Table III):

Table III.

Sugar	b	S.D.$_c$	S.D.$_c$
Glucose	0·340	0·0071	0·0033
Fructose	0·341	0·0067	0·0034
Invert sugar	0·338	0·0053	0·0028
Maltose	0·455	0·0077	0·0033

The figures show that by this rationalisation the standard deviation of calculated from observed is not appreciably increased. Hence the a factor of 0·05 with the b factors given in the above table are accepted as the standard values for the experimental conditions used. It will be seen that the fructose and glucose factors differ by less than 0·3 %, a fact of obvious advantage. The slightly lower value obtained for invert sugar will be discussed later.

The value S.D.$_e$ in Table III is the standard deviation in mg. sugar of the mean of four repeated determinations. It was calculated for each sugar from the expression:

$$\text{S.D.}_e = b\sqrt{\Sigma d/(f \times n)},$$

where d is the deviation in cc. $N/100$ thiosulphate from the mean of four repeated determinations, f is the total number of degrees of freedom (3 for each set of 4 repeated determinations), n the number of repeated determinations in each set (4), and b is the thiosulphate conversion factor for the sugar in question. Although the deviation from calculation (S.D.$_c$) is rather greater than the error due to successive estimations (S.D.$_e$), the scatter of the deviations about the sugar-thiosulphate lines calculated from the standard values of a and b does not suggest any significant departure from linearity. Moreover, it is clear that the linear relation fits the observations to within 0·5 % of the mean amount of sugar estimated (1·5 mg.).

Significance of the "a" factor.

A factor corresponding to the a factor (*i.e.* reduction curves not apparently passing through the origin) is common in copper reduction methods but had not been observed previously with the ferricyanide method; Hanes's curves, obtained for a sugar volume of 5 cc., passed through the origin. This fact, for 5 cc. sugar volumes, has been confirmed by the present authors so that the factors giving rise to a cannot be due to differences in personal technique. A confirmation of this fact is afforded by the agreement as to the value of a for 10 cc. sugar volumes obtained by the authors carrying out independent series of estimations. It might be expected that, using 15 cc. sugar volumes, a higher value for a would be obtained. Actually using such a volume it was impossible to obtain results sufficiently constant to be able to give any definite value to a under these conditions; values between 0·02 and 0·04 were obtained. This may be due to several causes—inequality of the initial stages of boiling, too high dilution of ferricyanide, *etc.*

The existence of factor a may mean (a) that some constant amount of sugar is destroyed before oxidation by ferricyanide, or (b) that a definite amount of re-oxidation of the ferrocyanide takes place during boiling, or (c) that auto-reduction of the ferricyanide occurs in water but not in the presence of sugar. This latter possibility seems hardly feasible in view of the identical water blank values obtained with 5, 10 and 15 cc. of water in the cold or with boiling for the standard time, while varying the volume of sugar solution does vary the value of a. Some evidence in favour of (b) is given by

the fact that Hanes found a somewhat low observed thiosulphate value when the sugar solutions were saturated with oxygen.

Thus it is difficult to find the theoretical significance of factor a, but from the point of view of actual sugar estimations it hardly warrants further investigation; indeed the factor may be neglected without introducing appreciable error so long as the amount of sugar to be estimated does not fall below 0·75 mg. Where a higher degree of accuracy is essential, and for amounts of sugar below 0·75 mg., the experimentally determined value of 0·05 cc. $N/100$ thiosulphate is quite satisfactory. To avoid error the factor is best applied by adding 0·05 to the water blank value, expressed as $N/100$ thiosulphate, when the requisite b factor may be applied directly.

Mixtures of sugars.

From the linearity of the relation between sugar and thiosulphate it is clear that a unit amount of sugar gives a constant reduction of ferricyanide in all parts of the scale investigated. It is essential to ascertain whether, in mixtures of sugars, one unit of each sugar will bring about a constant amount of ferricyanide reduction quite independent of the ferricyanide reduction caused by the other sugars. This was tested by comparing the observed thiosulphate value for mixtures of sugars with the simple sum of the calculated values for the component sugars. The results obtained are summarised in Table IV.

Table IV.

Sugar mixture (mg. sugar)				cc. $N/100$ thiosulphate	
Glucose	Invert sugar	Fructose	Maltose	Observed	Calculated
0·50	—	—	0·50	2·56	2·52
1·00	—	—	1·00	5·12	5·08
0·80	2·00	—	1·00	10·47	10·45
0·64	3·20	—	—	11·30	11·30
—	1·25	—	1·25	6·41	6·40
0·424	—	1·972	—	6·94	6·97
1·060	—	1·233	—	6·68	6·68
0·530	—	1·972	—	7·27	7·29
1·500	—	1·500	—	8·74	8·76

From these results it is clear that each sugar behaves towards ferricyanide as if the others were absent. However, the case of invert sugar considered as a mixture of equal parts of glucose and fructose is an apparent exception to this generalisation since the b factor, 0·338, is 0·0025 lower than the mean for glucose and fructose, 0·3405. "Synthetic" invert sugar (equal amounts of glucose and fructose) gives the expected factor as can be seen from the results for such an "invert sugar" solution given in Table IV. This suggests that the reducing power of invert sugar (as prepared by hydrochloric acid inversion of sucrose) with respect to ferricyanide is slightly higher than the expected additive reducing power of glucose and fructose. To suggest a reason for this would be pure speculation, although one obvious but unexpected possibility is that the acid present when the glucose and fructose are being formed from

sucrose might in some way slightly affect the reducing power of the two sugars. However, on treatment of a mixture of equal parts of glucose and fructose with 2·5 % hydrochloric acid under the conditions given by Hinton and Macara [1927] for the preparation of invert sugar from sucrose, the ferricyanide reducing power of the glucose and fructose was found not to have been affected in the slightest, the theoretical reducing value being found using the glucose-fructose *b* factors; see Table V.

Table V.

Glucose + fructose (mg.) (equal amounts)	Reducing value in cc. *N*/100 thiosulphate		
	Observed	Calculated for *b* factor for glucose + fructose	Calculated for *b* factor for invert sugar
2·134	6·23	6·22	6·27

Thus the possibility of hydrochloric acid affecting the reducing power of glucose and fructose under the conditions of sucrose inversion employed is ruled out. That the Hinton and Macara method for sucrose hydrolysis gives similar results to the Lane and Eynon method used in the invert sugar standardisation will be seen from Table VII, since the use of the *b* factor obtained for invert sugar prepared by the latter method gives agreement between actual and observed amounts of invert sugar prepared by the former method.

In addition to investigating the effect of one reducing sugar on another, the effect of the presence of sucrose was also investigated, since in copper reduction methods the presence of this sugar affects the reducing value obtained for reducing sugars. As will be seen from the results given in Table VI, the ferricyanide method is not appreciably affected by the presence of sucrose.

Table VI[1].

	Sucrose (mg.)	cc. *N*/100 thiosulphate
Glucose approx. 1·0 mg. (constant)	0·5	2·92
	1·0	2·92
	2·0	2·92
	4·0	2·94
Invert sugar approx. 1·0 mg. (constant)	0·5	2·94
	2·5	2·95

Fructose and maltose results are also unaffected by the presence of sucrose.

The fact that the total reducing value of a mixture of sugars, as estimated by the modified ferricyanide method described in this paper, is the simple sum of the reducing values of the component sugars and is not affected by the concentration of sucrose should give the method a great advantage over copper reduction methods in the estimation of the individual sugars present

[1] Note added July 29th. The authors have since found that sucrose alone does not cause any appreciable reduction of ferricyanide under the conditions of the modified Hanes estimation; this confirms the advantage of this method over copper reduction methods.

in, for example, leaf extracts. In the simplest case, the estimation of sucrose in a mixture of sugars, the increase in the reducing value following inversion may be converted directly into mg. invert sugar, using the appropriate factor (0·338). The invert sugar used for standardisation was prepared by hydrochloric acid inversion in the cold. In order to test whether the use of other methods of inversion would affect the ferricyanide reducing value, estimations of sucrose were carried out (1) by the citric acid inversion method and (2) by the hydrochloric acid inversion for 12 minutes at 60° as suggested by Hinton and Macara [1927].

(1) Inversion was carried out by 10 % citric acid in a boiling water-bath for 15 minutes, cooling and neutralising the solution with 5*N* NaOH; the requisite amount of NaOH was previously found on a separate amount of sugar-citric acid solution, using bromothymol blue indicator. The solution was then made to a definite volume and an aliquot taken for a reducing value estimation. It was found unsatisfactory to take 10 cc. of the neutralised solution for this estimation, for if this was done, the citrate present interfered with the precipitation of the potassium zinc ferrocyanide causing a shifting, indefinite end point, especially in the case of small amounts of sugar. It was found possible, however, to overcome this difficulty by taking only 5 cc. of inversion liquid and 5 cc. of distilled water, and then adding to the solution after the boiling with ferricyanide and cooling, in addition to the usual solutions, 1 cc. of 15 % zinc sulphate solution. The smaller amount of citrate present combined with the extra excess of zinc sulphate, and allowed of complete precipitation of the potassium zinc ferrocyanide, so that the thiosulphate titration end point was once more fixed and definite. The results obtained are given in Table VII.

(2) By the Hinton and Macara method, inversion was carried out by approximately 2·5 % hydrochloric acid in a water-bath at 60° for 12 minutes, agitating the liquid during the first 3 minutes of inversion. The solution was then neutralised with 5*N* NaOH and made to a definite volume; aliquots were taken for the determination of the reducing value. In both the citric and hydrochloric acid methods of inversion, control experiments were carried out, the sugar solution being replaced by distilled water. These controls were treated in a manner identical with that used for the sugar solutions and

Table VII.

	mg. sugar		% deviation observed from present
	Present	Observed	
Citric acid hydrolysis:			
Sucrose (as invert sugar)	0·263	0·267	1·5
,,	1·315	1·324	0·7
,,	2·632	2·670	1·4
HCl hydrolysis:			
Sucrose (as invert sugar)	1·894	1·894	Nil
,,	1·067	2·140	0·3
Glucose, fructose (equal amounts)	1·067		

the water blank (W.B.) value used for the reducing value determinations on the invert sugar solutions was obtained from these controls.

In order to confirm the validity of the hydrochloric acid method of inversion in sugar mixture determinations, an inversion was also carried out in the presence of equal amounts of glucose and fructose.

The results of the above determinations are given in Table VII.

Thus for estimation of sucrose as invert sugar by the ferricyanide reduction method inversion by 2·5 % hydrochloric acid for 12 minutes at 60° gives accurate results, while citric acid inversion gives results which are appreciably high. These high results are probably partly due to the effect of the citrate on the potassium zinc ferrocyanide. The presence of hydrochloric acid does not affect the reducing power of the other reducing sugars under the conditions of sucrose inversion employed. The hydrochloric acid method has, therefore, been adopted by the authors, although invertase inversion methods are being investigated to ascertain whether invert sugar prepared by this latter method gives a similar b factor to the one obtained on acid inversion.

For the hydrolysis of maltose in the presence of fructose or sucrose no really satisfactory method of acid hydrolysis is as yet available and investigations on this point and on the use of maltase are being carried out, especially in relation to sugar estimations in plant extracts.

For mixtures of glucose and fructose the modified Hanes method combined with iodimetric estimation gives easily calculated and accurate results.

The application of these two methods for the estimation of the individual sugars in simple mixtures of glucose, fructose and maltose and in leaf extracts is under investigation.

Summary.

1. A modification of the Hanes technique for the estimation of reducing sugars by oxidation with ferricyanide is described. This modification is shown to give a linear relation between the amount of sugar and the amount of ferricyanide reduced over a range of 0·2 to 3·0 mg. in the case of glucose, fructose and invert sugar, and of 0·2 to 3·5 mg. in the case of maltose. By statistical treatment the linear relation is found to be accurate to 0·0067 mg. sugar (mean value for the four sugars), *i.e.* 0·5 % of the mean amount of the sugars estimated.

2. Expressing the relation between amount of sugar and the amount of ferricyanide reduced in terms of $N/100$ thiosulphate, the following relation is found:

$$\text{Sugar} = b\,(\text{observed thiosulphate} + a),$$

a having the value 0·05 cc. $N/100$ thiosulphate for all the sugars investigated; factor b is shown to be, for glucose 0·340, for fructose 0·341, for invert sugar (prepared by HCl inversion of sucrose) 0·338 and for maltose 0·455.

3. In a mixture of sugars the ferricyanide reducing power of one sugar is shown to be unaffected by the presence of the others. The presence of

sucrose does not affect the reducing value of the other sugars present in a mixture.

4. Invert sugar, prepared by acid hydrolysis of sucrose, has a slightly higher reducing value than a synthetic mixture composed of equal amounts of glucose and fructose.

5. For the estimation of sucrose, inversion by 2·5 % hydrochloric acid for 12½ minutes at 60° (after Hinton and Macara) followed by determination of ferricyanide reducing power is found to give accurate results, while citric acid inversion gives results which are too high.

6. The application of the ferricyanide reduction method to the estimation of individual sugars in plant extracts is discussed.

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CXIX. THE CARBOHYDRATE COMPLEX OF THE SERUM PROTEINS.

II. IMPROVED METHOD FOR ISOLATION AND RE-DETERMINATION OF STRUCTURE. ISOLATION OF GLUCOSAMINODIMANNOSE FROM PROTEINS OF OX BLOOD.

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In a previous communication [Rimington, 1929] the isolation was reported of a nitrogen-containing polysaccharide from both the albumin and globulin of horse's blood-serum. The yield from each protein was about 2 %. It was also shown that this non-reducing complex gave rise, upon acid hydrolysis, to glucosamine and a hexose which was identified as mannose. On the basis of analytical figures, more particularly the nitrogen content of the isolated complex, it was concluded that the simple structure consisted of 1 molecule of glucosamine associated with 1 molecule of mannose and a structural formula was proposed which would account both for the lack of reducing property and for the liberation of nitrogen from the amino-group when acted upon by nitrous acid.

Shortly after this paper was published, a communication appeared from Levene and Mori [1929] who claimed to have isolated a carbohydrate complex from ovalbumin and ovomucoid in which 1 molecule of glucosamine was associated with 2 molecules of mannose. From molecular weight determinations by the diffusion method, Levene and Rothen [1929] came to the conclusion that the complex was a polymeride consisting of 4 units of the trisaccharide, *i.e.* 12 hexose residues in all. These workers found that relatively prolonged hydrolysis by baryta was necessary to free the carbohydrate completely from accompanying protein material.

Since the carbohydrate isolated from egg-protein by Levene and Mori was in many respects similar to that obtained from serum, it was thought to be desirable to reinvestigate the conditions under which the latter could be obtained in pure form and to establish with certainty its ultimate di- or trisaccharide nature.

It was found that by further prolonged hydrolysis of the preparations previously reported upon, some nitrogenous material was eliminated, leaving

a substance with a nitrogen content which became constant at 2·7 % agreeing with that required by a trisaccharide structure. The impurity present in the original preparations was only removed with difficulty and by the application of relatively vigorous methods. It proved to consist largely of histidine. By comparison of the analytical figures for histidine with those required by the di- and tri-saccharide formulae it will readily be appreciated that its presence in small quantity might be overlooked. The pure carbohydrate has now been

	C	H	N
Histidine	46·42	5·85	27·10
Disaccharide (glucosamine + 1 mannose)	42·21	6·79	4·10
Trisaccharide (glucosamine + 2 mannose)	42·94	6·56	2·78

obtained from the serum proteins of the horse and ox. In each case the composition indicates a trisaccharide nature and as far as it is possible to tell, the preparations appear to be identical.

Levene and Mori found that from ovomucoid as much as 5 % of carbohydrate could be isolated and concluded, therefore, that this protein was the source of the material isolated from crystallised ovalbumin preparations. Although the carbohydrate content of egg-white appeared to decrease on crystallisation of the protein it is difficult to believe that a three times recrystallised preparation could have contained as much as 5 % of associated ovomucoid, the figure demanded by the quantities of carbohydrate isolated from each individual protein (5·1 % from ovomucoid, 0·26 % from thrice recrystallised ovalbumin).

Sørensen [1930] has shown that egg-albumin dissociates to some extent in solution, so that by successive recrystallisation fractions are continually being left behind in the mother-liquors which differ from the crystalline phase by possessing a relatively higher solubility; these fractions also show differences in chemical composition. It might well be that, by this process, carbohydrate-rich fractions are eliminated from the bulk of the protein, a hypothesis which would explain the decreasing carbohydrate content observed by Levene and Mori, of repeatedly recrystallised egg-albumin more readily than the supposition that only ovomucoid contains carbohydrate, the albumin being free from carbohydrate.

In the case of blood it is known that there is present in the serum a protein to which the name seromucoid has been given [Zanetti, 1897, 1903; Bywaters, 1909]. Although present to the extent of only 0·1 to 0·5 g. per litre of blood, seromucoid has been credited with a high carbohydrate content, 20 % or more, which if true, would mean that the sugar so found, accounted for as much as 10 % of the total protein-sugar of the blood.

An investigation of this protein is in progress and will be published shortly. It appears to contain a carbohydrate complex similar to that present in the albumin and globulin and in quantity approaching 25 % (determined colorimetrically by comparison with glucose and recalculated in terms of glucosaminodimannose).

For reasons similar to those outlined above in the case of egg-proteins, it is considered unlikely that the carbohydrate isolated from serum-albumin and globulin preparations was in reality derived from admixed seromucoid. The quantity obtained, about 2 % of the weight of the protein, was too great to render this likely, moreover evidence is presented below which proves all but conclusively that, in the case of horse serum-globulin, the carbohydrate enters into the protein molecule as a true constituent to the extent of approximately 3·7 %. Final solution of the question may be deferred until the identity of the seromucoid carbohydrate has been established. It is noteworthy, however, that Lustig and Haas [1931] have recently investigated the carbohydrate content of blood-albumin and globulin preparations obtained by an elaborate series of fractionations and have recorded values which strongly support the view that carbohydrate enters into each of these proteins as an integral constituent.

That the carbohydrate complexes isolated from the proteins of horse and ox bloods appear to be identical does not, at first sight, appear to support the view put forward in the first paper of this series [Rimington, 1929] that the polysaccharide may play an active part in immunity reactions. This point is, however, under consideration and may be the subject of further communications. That what is apparently the same carbohydrate appears in so many different situations and species is undoubtedly a remarkable fact.

With regard to the structure of the trisaccharide complex there seems to be no reason why the argument originally put forward in support of a betaine-like linkage involving the nitrogen atom of the glucosamine molecule should not be equally applicable to the case of three hexose residues united together. Only exhaustive methylation and hydrolysis would show the point of attachment of the constituents. The slow liberation of nitrogen from the amino-group indicates however, as formerly, a union similar to that existing in glucosamine methyl glucoside. Karrer [1930] has put forward a similar view to account, on structural lines, for the known properties of chitosan. More recently Bertho *et al.* [1931] have discussed this question in relation to their work upon synthetic N-acyl derivatives of glucosamine and suggest that proximity of the $—NH_2$ to the —CHO group may alone be sufficient to cause abnormality in the reactions of the latter. The lack of activity of the aldehydic group towards the usual reagents would be interpreted by these authors as due to steric hindrance rather than to the existence of a true betaine grouping.

As to how the carbohydrate complex is united to the protein, no suggestion is at present ventured. It is however certain that, whatever the linkage, it is of a type that is markedly resistant to prolonged alkaline hydrolysis. Different proteins appear to vary in this respect, since in the present case 30 to 36 hours' boiling with 10 volumes of 10 % baryta was found necessary to eliminate all accompanying material from the blood-serum carbohydrate, whilst Levene

and Mori found that pure material could be obtained from the various egg-proteins after only 7 or 8 hours' boiling with the same reagent.

Unfortunately, appreciable destruction of the carbohydrate occurs during the more extended periods of hydrolysis with consequently lower yields, and no way has been found up to the present of obviating this loss of material.

Experimental.

As a result of previous work the conclusion had been drawn that the carbohydrate complex obtainable from serum-albumin and globulin was a disaccharide structure (possibly polymerised) containing one molecule of glucosamine and one of mannose and having therefore a nitrogen content of 4·1 %. Preparations isolated after 3 hours' hydrolysis of the protein by 10 % baryta appeared to approximate fairly closely in composition to this figure, provided glacial acetic acid had been used in the final stages as a precipitating reagent. If this treatment, which involved considerable loss of material, was omitted, nitrogen contents prevailed of 4·5 % and upwards. As a result of the investigations detailed below it appears that the carbohydrate is in reality a polymeride of a trisaccharide containing 2 molecules of mannose associated with every one of glucosamine, and thus possessing a nitrogen content when pure of 2·78 %. Certain modifications have been made in the method of isolation, details of which will be found below.

Preparation of carbohydrate from mixed proteins of horse-serum.

100 g. mixed albumin and globulin were boiled under reflux with 1 litre of 10 % barium hydroxide for 8 hours. (This proved to be too short a time; in later experiments 30–36 hours' hydrolysis was allowed.) To the filtered solution, sufficient 50 % sulphuric acid was added to render the reaction faintly acid to litmus and the barium sulphate removed by filtration and washed. 150 cc. of saturated basic lead acetate were added and then saturated baryta until maximal precipitation was attained. After removal of this precipitate on the Büchner funnel the filtrate was found still to give a faintly positive Molisch test. A further 75 cc. of basic acetate was therefore added and baryta as before. After this precipitation the solution was carbohydrate-free. The lead-barium precipitate was ground into a thin cream with water and sufficient 33 % acetic acid added to make the mixture faintly acid to litmus. The bulk of the precipitate dissolved leaving an orange-yellow material. If this gives a positive Molisch reaction, positive addition of acetic acid is cautiously continued until all the carbohydrate is in solution. The undissolved residue is filtered off and to the yellow-brown solution (allowing the acid solution to stand for 12–24 hours is recommended, since during this time a deposit of dark orange material settles out which can be filtered off and discarded) is added 3·0 cc. of basic lead acetate followed by baryta as before. The carbohydrate-lead compound is soluble in excess of baryta, so care should

be taken during the addition. After repetition at least three times of the solution in acetic acid and reprecipitation by baryta, the final lead-baryta precipitate is suspended in water and decomposed by a stream of carbon dioxide at 50–55°. Re-suspension of the lead carbonate and treatment with CO_2 is repeated until all the carbohydrate has passed into solution. To the combined liquids, a moderate excess of Hopkins's reagent (10 % $HgSO_4$ in 5 % H_2SO_4) is added and the mixture allowed to stand for several days. It is important that the total concentration of acid should not exceed 5 % whilst this precipitation is going on. As will be described later the material removed by mercuric sulphate has been shown to consist largely of histidine, and Calvery [1929] has shown that the histidine-mercury compound is soluble in sulphuric acid exceeding 5 % in strength.

After filtration lead and mercury are removed by hydrogen sulphide and the sulphuric acid by baryta. The solution is then concentrated *in vacuo* to a small bulk and the final elimination of SO_4 or Ba ions effected at this stage. Further concentration is performed in a vacuum desiccator over sulphuric acid, and to the syrup remaining is added about 20 cc. of dry methyl alcohol followed by an equal volume of ether. The precipitated carbohydrate is centrifuged, reprecipitated once or twice from concentrated watery solution and finally dehydrated by absolute alcohol and ether. Should the nitrogen content prove to be high, a further hydrolysis for 6–10 hours by 10 % baryta is necessary, followed by isolation as above, but in this case the basic lead acetate-baryta precipitation may be omitted.

Levene and Mori observed that pure material, from which all that was precipitable by mercuric sulphate had been removed, yielded nevertheless an additional precipitate with this reagent after further hydrolysis had been performed.

Precisely similar findings were encountered in the present case, which might be taken to indicate that the carboxyl group of the histidine, chief constituent of the mercury precipitate, is involved in some way in the union with the carbohydrate. Too great weight is not to be attached to this interpretation; it is put forward rather as a tentative suggestion as to how the complex may be united to the protein.

A yield of 3·5 g. of carbohydrate having a nitrogen content of 5·23 % was obtained. Since it was obviously still impure a further 8 hours' hydrolysis by baryta was performed and the isolation repeated. Yield 0·9 g. having 3·48 % of N; after 10 hours' more hydrolysis, 0·38 g. of pure material was obtained with 2·79 % of N.

In order to demonstrate that the nitrogen content remained constant at this figure and that the material was therefore pure, a further hydrolysis for 10 hours in 10 % baryta was carried out and 0·18 g. of material recovered with 2·66 % of N.

The steps in this purification, the yields and analytical data are summarised on p. 1067.

100 g. mixed proteins of horse-serum.

Hydrolysis (hr.)	Yield g.	Ash-free C	Ash-free H	Ash-free N*	Reaction with diazobenzenesulphonic acid $HgSO_4$ ppt.	
8	3·5	44·30	6·56	5·23	+	+ +
8 more	0·9	41·50	7·05	3·48	+	±
10 ,,	0·3	41·58	6·39	2·79	−	−
10 ,,	0·1	41·91	6·31	2·66	−	−
Calculated for $C_{18}H_{33}O_{15}N$		42·94	6·56	2·78		

* Micro-Kjeldahl by Mr A. R. Colwell, Cambridge. It was found that determinations on the same material by micro-Dumas gave constantly high results. C and H micro-determination by Dr Schoeller, Berlin.

Since histidine is the most tenaciously adhering impurity, applying the diazo-reaction with diazotised sulphanilic acid affords an indication of the progress of purification. The pure carbohydrate gives no reaction, neither does any precipitate form when mercuric sulphate reagent is added to its solutions after a further hydrolysis. Either test may be used to check the purity of material during the course of isolation.

Properties of the pure carbohydrate.

As precipitated from watery solution by alcohol and ether, the carbohydrate complex is obtained as a hygroscopic, creamy-white amorphous material. Its solutions are pale straw-coloured, viscous when concentrated, and show no measurable optical activity. They do not reduce Fehling's solution until after acid hydrolysis. The absence of optical activity may be due to racemisation during the prolonged hydrolysis. In the Van Slyke apparatus nitrogen is slowly evolved indicating the presence of a free amino-group as discussed previously. After acid hydrolysis no sugar except mannose and glucosamine could be detected.

Isolation from mixed proteins of ox-serum.

100 g. of the heat-coagulated mixed proteins from ox-serum[1] was boiled under reflux with 10 times the quantity of 10 % baryta. The hydrolysis was allowed to proceed uninterruptedly for 30 hours, after which the process of isolation was gone through precisely as described for the horse-serum carbohydrate. A yield of 1·1 g. was obtained. The N content was 3·72 %, thus indicating that nitrogenous impurities were still present. The whole of the material was therefore again hydrolysed with 10 % baryta for 12 hours. In carrying out the isolation the entire process was repeated without omission of any of the stages. Yield 0·48 g. having N 2·70 %. The full analyses were as follows:

	Ash-free C	Ash-free H	Ash-free N	Yield g.
Initial 30 hours' hydrolysis	42·94	6·31	3·72	1·1
After further 12 hours' hydrolysis	44·65	6·53	2·70	0·45
Calculated for $C_{18}H_{33}O_{15}N$	42·94	6·56	2·78	—

In general properties, the material was similar to that isolated from horse-serum proteins.

[1] For the preparations of ox-globulin and albumin I am indebted to Messrs Boots Pure Drug Co., Ltd., Nottingham, who kindly placed this quantity at my disposal.

Detection of histidine among the impurities present in the crude preparation.

As mentioned previously, it was found that crude preparations of the carbohydrate still contained nitrogenous impurities which were not precipitable by Hopkins's reagent, but which could be liberated by further boiling of the complex with baryta and were then removable as their mercury salts.

A small quantity of the mercury precipitate was suspended in water and decomposed by prolonged saturation with hydrogen sulphide gas. After removal of the sulphide, which showed a marked tendency towards the colloidal form, a portion of the solution was subjected to the following tests.

Biuret reaction	Very faintly positive; pink
Ninhydrin reaction	Intense
Millon reaction	Negative
Glyoxylic reaction	Positive, but atypical
Molisch reaction	Faintly positive, but accompanied by a dark brown colour.
Diazo reaction (sulphanilic acid)	Intense, deep red
Diazo reaction (sulphanilic acid) as modified by Totani	Clear golden yellow after addition of hydrogen peroxide
Phosphotungstic acid	Immediate precipitate, soluble on warming but reappearing as the solution cooled

The remainder was purified through the phosphotungstate and then gave all the typical reactions of histidine; the quantity present, however, was not great. The filtrate from the histidine phosphotungstate no longer gave any reaction with ninhydrin. It would appear from the above that histidine constitutes the major part of the impurity in crude carbohydrate preparations. It must be in some way combined with the polysaccharide since acid mercuric sulphate fails to precipitate it from solution.

Determination of the carbohydrate content of the serum-proteins by colorimetric methods.

Since isolation of a substance from a complex mixture is invariably attended by some degree of unavoidable loss, an attempt was made to determine the true carbohydrate content of the serum-proteins by an indirect means.

Methods available for the determination of carbohydrate materials fall into two classes, those depending upon the reducing property of the free aldehydic group and colorimetric methods. The former are inapplicable in this case since the conditions under which the protein gives rise to reducing sugars (acid hydrolysis) are also those favourable to secondary reactions whereby carbohydrate and amino compounds combine to form "humin" [see Gortner, 1916].

A colorimetric method has been proposed by Dische and Popper [1926] based upon the interaction between carbohydrates and indole in the presence of 70 % (by volume) sulphuric acid as condensing agent. The colour value is not strictly proportional to the quantity of sugar but this deviation is easily

corrected for[1]; moreover Dische and Popper showed that the same value was given by sugars whether in the free or combined state, thus starch and glycogen could be matched directly against glucose. Mannose gives only about 65–70 % of the colour of glucose whilst glucosamine does not react at all. The method is applicable to the determination of the combined carbohydrate in proteins and in the present writer's hands has proved most serviceable. A very similar method is that proposed by Tillmans and Phillipi [1929] who employ orcinol together with 60 % (by volume) sulphuric acid. This was the method employed by Lustig and Haas [1931] in their examination of serum-globulin subfractions. Mannose reacts quantitatively like glucose, but the amino-sugars fail to give any colour, as in the indole method.

From a comparison of the two methods one would expect that the pure glucosaminodimannose complex would have a higher glucose equivalent by the latter than by the former, which is indeed the case. Further support is thus offered to the structure already assigned to the complex. In the investigations with proteins, Dische and Popper's method was more frequently employed although both this and Tillman and Phillipi's orcinol method yielded the same result (see below).

Pure glucosaminodimannose (N, 2·70 %).

1·675 mg. was dissolved in 5 cc. of water and 1 cc. aliquots compared with a 0·02 % glucose solution.

By Dische and Popper's method 1·675 mg. polysaccharide≡0·6695 mg. glucose. Hence 39·96 % of the colour of glucose.

By Tillman and Phillipi's method 1·675 mg. polysaccharide≡1·099 mg. glucose. Hence 65·60 % of the colour of glucose.

Assuming mannose to react quantitatively, theory would require 66·6 %.

Ox-serum globulin*:

Dissolved 0·1024 g. in 5 cc. of 0·05 *N* sodium hydroxide.

By micro-Kjeldahl N = 11·825 mg. in all.

Total carbohydrate (on 1 cc. aliquot) = 1·468 % as glucose by Dische and Popper's method, *i.e.* 3·67 % of the protein when calculated as glucosaminodimannose.

Total carbohydrate (on 1 cc. aliquot) = 2·279 % as glucose by Tillman and Phillipi's method, *i.e.* 3·47 % of the protein when calculated as glucosaminodimannose.

* For this preparation I am indebted to Messrs Boots Pure Drug Co., Ltd., of Nottingham. The total nitrogen was taken as 15·85 % in calculating the carbohydrate present.

The agreement between the two methods is satisfactory. The preparation of horse-serum globulin utilised by the present writer in the experiments culminating in the isolation, for the first time, of the carbohydrate complex was now examined. This protein sample had been very thoroughly purified [see Rimington, 1929], however, its carbohydrate content was found to be very similar to that of the ox-globulin above, and moreover this figure was found to suffer no decrease after thorough washing of the suspended protein particles, a treatment which would, undoubtedly, have removed any seromucoid still adhering.

[1] An empirically determined correction curve was employed, not the formula of Dische and Popper.

Horse-serum globulin, highly purified preparation.

Dissolved 0·1005 g. in 5 cc. of 0·05 *N* sodium hydroxide.

By micro-Kjeldahl N = 13·873 mg. in all.

Total carbohydrate (on 1 cc. aliquot) = 1·3 % as glucose, *i.e.* 3·74 % of the protein when calculated as glucosaminodimannose.

A further sample of about 0·1 g. of protein was placed in a stoppered centrifuge tube with 30 cc. of distilled water and subjected to vigorous mechanical shaking for 6 hours. After centrifuging and washing it was dissolved in dilute alkali as above and the carbohydrate content redetermined.

By micro-Kjeldahl N = 14·755 mg. in all.

Total carbohydrate (on 1 cc. aliquot) = 1·73 % as glucose, *i.e.* 4·32 % of the protein when calculated as glucosaminodimannose.

It seemed of interest to compare the carbohydrate content of the protein as determined colorimetrically with the actual yield obtainable by isolation. From the above figures it is clear that this sample of horse-serum globulin contained about 3·8 % of glucosaminodimannose. After an initial 3½ hours' hydrolysis there had been isolated 3·91 % of material having a nitrogen content of 4·92 %. As it is now known that the substance when pure has 2·78 % N, and that the major portion of the impurity is in all probability histidine (N, 27·10 %) it is permissible to deduct 9 % by weight corresponding to this amino-acid and to assume that the remaining material would be pure glucosaminodimannose. The corrected yield works out at 3·6 % in very good agreement with the content of 3·8 % determined colorimetrically. Of course, much loss is incurred if the crude material is boiled for any considerable length of time with baryta in order to effect further purification, so that yields of pure material as high as this are not directly attainable. In the previous investigation the yield had dropped to a little over 2 % by the time the nitrogen content was reduced to 4 %.

It seems quite certain that the carbohydrate content of the serum-proteins is considerably higher than has previously been supposed.

Summary.

1. A reinvestigation of the conditions under which the carbohydrate complex of the serum-proteins can be isolated in the pure state has led to modifications in the scheme of separation originally proposed.

2. It also appears that the carbohydrate is composed of an association of one molecule of glucosamine with two of mannose, being thus similar to that obtainable from egg-proteins.

3. The impurity accompanying the original preparations has been shown to consist largely of histidine. This amino-acid appeared to be united chemically to the complex.

4. Glucosaminodimannose has been isolated from the mixed serum-proteins of both horse- and ox-blood.

5. Colorimetric methods have been used to determine the carbohydrate

content of proteins. By reference to the colour value of the pure complex, such figures are expressible directly as glucosaminodimannose. The values obtained by two different methods are in agreement.

6. Serum-globulin appears to contain approximately 3·7 % of carbohydrate.

7. This figure is not decreased by thorough washing of the coagulated protein and is taken therefore to represent carbohydrate in actual structural combination. Decreasing values obtained after repeated crystallisation of native proteins should be interpreted with caution, since, it is not improbable that dissociation of the component systems comprising the protein takes place in solution, the carbohydrate-rich fractions being left in the mother-liquors.

My thanks are due to Mr A. T. King for his continued interest in these studies and to Mr A. R. Colwell of the Biochemical Laboratory, Cambridge, for his kindness in performing numerous micro-Kjeldahl determinations.

I also wish to acknowledge the benefit I have derived from correspondence with Prof. P. A. Levene upon the subject of the presence of carbohydrate groups in the serum- and egg-proteins.

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CXX. THE RELATION BETWEEN TOTAL SULPHUR AND CYSTINE CONTENT OF CERTAIN SAMPLES OF RABBIT FUR.

By JOHN BARRITT and CLAUDE RIMINGTON.

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(*Received June 22nd, 1931.*)

In a recent study of the effect of diets supplemented with cystine upon the composition of the keratin of rabbit fur, large variations, which it was concluded were seasonal, were found to occur in the total sulphur content of the fur of both control and experimental animals [Barritt, King and Pickard, 1930].

It was considered to be of interest to ascertain whether these increased sulphur values were represented wholly by cystine, since it has been shown [Rimington, 1929, 1, 2] that almost the whole of the sulphur in wool and various animal hairs can be accounted for in this form.

The results indicate that within slight limits, possibly of experimental error, the sulphur in all the samples examined can be accounted for in this form.

Some observations made upon the technique of colorimetric cystine determination emphasise, as detailed below, the instability of this amino-acid and the necessity of observing certain experimental precautions if low values are to be avoided.

Experimental.

The samples of rabbit fur were obtained from a number of Angora rabbits fed on a normal diet to which various additions of cystine were made during an interval of 84 days [Barritt, King and Pickard, 1930]. The material was sampled and purified as previously described [Barritt and King, 1926, 1929].

Approximately 1 g. of fur was hydrolysed with 25 cc. of 20 % hydrochloric acid for 5½ hours. During the initial stages of hydrolysis there was a tendency for violent bumping to occur. Any slight loss of material was not, however, of serious moment since only comparative values were required of total sulphur by the modified Benedict-Denis method, and of cystine by the Folin-Marenzi [1929] method, in the hydrolysate. The values for total sulphur in the samples were obtained by the Carius method [Barritt, King and Pickard, 1930].

Two 5 cc. samples of the hydrolysate were taken for total sulphur determination as described by Rimington [1930, 1]. For determination of cystine a 5 cc. sample of the hydrolysate was diluted to approximately 42 cc. and 5 cc. of 20 % sodium hydroxide were added slowly with constant shaking, this being sufficient to reduce the acidity to approximately $N/10$. The mixture was made up to 50 cc., filtered and 2 cc. portions of this solution taken for the colorimetric procedure using urea to prevent turbidity [Rimington, 1930, 2].

The detailed results of Table I show that the increased total sulphur of the second clip can be accounted for by the higher cystine content of these furs.

Table I.

	Rabbit	Total sulphur (Carius)	Cystine in 5 cc. of hydrolysate (Folin-Marenzi) (mg.)	Cystine in 5 cc. of hydrolysate calculated from determination of total sulphur (Benedict-Denis) (mg.)	Difference (mg.)	Difference (%)
Controls	103 B	5·10	32·5	32·8	−0·3	−1·0
	103 A	5·05	—	36·3	—	—
	243 B	4·64	33·7	35·0	−1·3	−3·7
	243 A	4·98	37·4	36·7	+0·7	+1·9
	302 B	4·12	30·7	30·7	0	0
	302 A	4·79	41·1	41·75	−0·65	−1·6
0·1 g. cystine per day	63 B	4·93	47·1	46·0	+1·1	+2·4
	63 A	5·11	39·7	39·6	+0·1	+0·25
	235 B	4·77	32·3	32·2	+0·1	+0·3
	235 A	4·91	36·4	36·5	−0·1	−0·3
0·2 g. cystine per day	193 B	4·92	38·7	38·9	−0·2	−0·5
	193 A	5·02	41·1	41·8	−0·7	−1·7
	238 B	4·85	35·1	35·4	−0·3	−0·85
	238 A	5·05	37·4	36·4	+1·0	+2·7
	253 B	4·57	36·7	37·2	−0·5	−1·3
	253 A	4·89	37·6	38·1	−0·5	−1·3

Samples marked B were clipped on April 18th, 1929.
Samples marked A were clipped on July 11th, 1929.

The effect of concentration during the neutralisation of the hydrolysate.

It was found advisable to adhere carefully to the above procedure for the colorimetric determinations since, otherwise, anomalous results were obtained. In particular it is essential that the hydrolysate should be largely diluted before addition of the alkali since neutralisation of a concentrated solution, even if carried out with every precaution against rise of temperature, is liable to lead to very appreciable destruction of cystine. In a particular example a 10 cc. sample of the undiluted hydrolysate was treated with 5 cc. of 40 % sodium hydroxide, added drop by drop, the vessel being immersed in cold water and kept continuously agitated. The mixture was filtered and of the almost colourless filtrate 5 cc. were taken for a determination of total sulphur

whilst another 5 cc. were diluted to 50 cc. and used for the colorimetric determination.

The results obtained were as follows:

Total sulphur (Benedict-Denis) equivalent to 19·2 mg. cystine/5 cc. filtrate.
Cystine sulphur (Folin-Marenzi) equivalent to 13·1 mg. cystine/5 cc. filtrate.

Or expressed as cystine in the original hydrolysate:

Total sulphur (Benedict-Denis) equivalent to 28·8 mg. cystine/5 cc. hydrolysate.
Cystine sulphur (Folin-Marenzi) equivalent to 19·6 mg. cystine/5 cc. hydrolysate.

When the hydrolysate was diluted in the normal way, as described above, the following values were obtained which show good agreement between total sulphur and cystine sulphur:

Total sulphur (Benedict-Denis) equivalent to 28·5 mg. cystine/5 cc. hydrolysate.
Cystine sulphur (Folin-Marenzi) equivalent to 28·6 mg. cystine/5 cc. hydrolysate.

It would appear that concentrated sodium hydroxide acting upon the undiluted hydrolysate causes a very appreciable destruction of cystine.

The authors' thanks are tendered to Mr A. T. King, Head of the Chemical Laboratories of the Association, for help and advice rendered during the course of their work.

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CXXI. NOTE ON THE SULPHUR-FREE NATURE OF MEDULLA IN BLACKFACE WOOL.

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(*Received June 22nd, 1931.*)

The presence of a medullated core is a well-known characteristic distinguishing the coarser (kempy) types of wool fibre from the fine merino type comprised only of scale and cortex.

Knowledge of the nature and amount of this medullary substance was confined to qualitative microscopic observation prior to the figures derived by Barker and King [1926] from a comparison of observed fibre diameters with those calculated from length, weight and density determinations.

The close agreement of the two methods, when non-medullated fibres are employed, falls off in proportion to the extent of medullation, but this circumstance provides a means of calculating approximately the relative proportions of medulla substance, air space, and non-medullated substance (cortex and scale) contained in a medullated fibre.

Blackface wool fibres were employed, as the remarkable regularity of both medulla diameter and total fibre diameter makes them eminently suitable for the measurements concerned.

From a series of measurements, detailed in the paper referred to, on typical medullated fibres (excluding heavily or only slightly medullated fibres) the following values were calculated:

mean percentage of air space to total fibre volume approx. 55·0 %;

mean percentage of air space to volume of medulla substance approx. 90 %.

Thus the average content of actual medulla substance in these fibres is 6·1 %.

Determinations of sulphur content [Barritt and King, 1926] of the bulk sample of moderately medullated fibres from which the fibres for the above measurements were drawn gave 3·33 %, while that for fine unmedullated fibres from the same portion of the fleece gave 3·82 %. From these values, making the assumption that the sulphur content of the cortex and scale portion is the same for both medullated and unmedullated fibres (*i.e.* 3·82 %) an equation relating the medulla channel and the sulphur content of the actual medulla substance can be derived as follows.

Page 1075, 5th para., last line:

for **5·5%** *read* **6·1%**

Let X = % volume of medulla channel in total fibre volume,

and Sm = sulphur content of medulla substance,

then $$\frac{X}{10}Sm + (100 - X)\,3{\cdot}82 = \left(100 - X + \frac{X}{10}\right)3{\cdot}33.$$

Hence $$490 = X\,(8{\cdot}23 - Sm).$$

Taking arbitrary values of Sm the following values of X are obtained:

Sm	X
0	59·5
1	67·8
2	78·6
3	94·0

The figure of 55 % obtained by Barker and King for the average air-space in the medullated fibres corresponds to ~~60·5~~ 61·1 % of medulla channel, which is in striking agreement with the calculated figure of 59·5 % assuming zero sulphur content in the medulla substance.

Reviewing the possible sources of error which might combine to make this agreement merely a coincidence, the actual measurements and sulphur determinations are regarded as of sufficient precision to preclude any appreciable error in the figure deduced.

The assumptions necessarily made are, firstly, that the density of the medulla substance is the same as that of the unmedullated fibre. Widely differing types of wool fibre have practically identical density values [King, 1926] and the density of dry gelatin, 1·34 [see Svedberg, 1930] and of protein matters generally is not very different from 1·30 for wool substance, whereas only a wide divergence would seriously affect the result.

Secondly, it has been assumed that if the scales and cortex differ in sulphur content, any variation in the relative proportions of scale and cortex will not seriously affect the sulphur value. This can only be decided when unscaled fibres [King, 1927] can be analysed for sulphur content.

Thirdly, alternatively to the assumption made that the sulphur content of the unmedullated portion is the same as of the non-medullated fibres, a generally lower value in all the components of the medullated fibre would of course equally explain the difference in actual sulphur values. The direct evidence advanced in the foregoing paper however, affords a very strong substantiation of the mathematical deduction that the sulphur content of medulla substance, if not actually *nil*, must be extremely low in comparison with that of scale and cortex.

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Page 1076, 1st para., 7th line:
for **60·5%** *read* **61·1%**

Page 1076, last para., 5th line:

CXXII. SULPHUR DISTRIBUTION IN THE COMPONENT STRUCTURES OF WOOL AND PORCUPINE QUILLS.

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(*Received June 22nd, 1931.*)

The views originally advanced by one of us [King, 1926] and in more detail by Barritt and King [1929] regarding the biological significance of sulphur variability in wool are, in brief, that, in the first place, "keratinisation" is the result of incorporation of cystine nuclei with the primary plasmic cell-forming material, the extent of this incorporation or "sulphur stimulus" being variable with different types of wool and with different genetic, pathological, and environmental factors, particularly, as regards the last named, diet and seasonal conditions. Further evidence of this variability has been published by Rimington [1929] and Barritt and King [1929] as opposed to the statement of Robertson and Marston [1929] that wool keratin has a constant sulphur content.

Recent work in these laboratories by Bonsma [1931] has established some evidence of correlation of sulphur content with diet and seasonal changes in the case of South African wool, and his discussion of its biological and technical significance is substantially an elaboration of the views already advanced [King, 1929].

Secondly, from indirect evidence, summarised in the first paper above mentioned (details of which are published for the first time in the accompanying note [Barritt and King, 1931]) it was concluded that "the medulla cells...are substantially devoid of sulphur" and that a biological deficiency in "sulphur stimulus" is a factor associated with presence of the medulla, unkeratinised by cystine incorporation, which constitutes the inner core of kempy fibres.

The essential evidence lies in the substantially lower sulphur content of medullated fibres as compared with non-medullated fibres from the same portion of the fleece, and, in view of the fact that recently Rimington [1931] found only comparatively small differences in this respect with certain types of wool, it appeared desirable to examine some hirsute tissue in which the medulla could be separated with some degree of accuracy and independently analysed.

For this purpose porcupine quills, morphologically analogous to wool fibre,

were employed. They were split with a knife, and the medullated core carefully scraped away from the cortex. Complete separation is impracticable on account of the radially disposed lamellae of cortical tissue (see Fig. 1*a*) but, while the medulla remained contaminated with the cortical lamellae, the cortex was obtained substantially free from medulla.

The samples were degreased with boiling benzene, washed in water, and dried to constant weight.

Analysis gave the following results[1].

Ash (whole quill) 0·79 %.

	Whole quill	Cortex	Medulla
Total sulphur	1·31	1·46	1·25
	1·38	1·53	1·23
Mean	1·35	1·50	1·24

The sulphur content for the whole quill is thus 10 % lower than that for the cortex. Typical values for non-kempy and kempy wool fibre (3·82 and 3·33 respectively) show a difference of 12·8 %, a figure of the same order. The actual weight of medulla substance in the wool kemp examined is approximately only 5 % of the total fibre weight. Unfortunately, as mentioned above, the failure to obtain complete separation of the porcupine medulla again precludes direct analytical evidence of its freedom from sulphur, but the microscopic appearance of its very attenuated structure of hollow cells indicates that its proportion by weight of the whole quill must be very small, and the results therefore are not inconsistent with those for medullated wool fibre.

Confirmatory evidence was sought from the action of specific staining agents on sections of both wool and quill.

Sodium plumbate solution (10 minutes treatment in the cold) rather disturbs the structure on account of its alkaline character, but shows clearly the location of the sulphur in the cortex (Fig. 1*b*).

The Sullivan reagent (applied by boiling the quill section in dilute hydrochloric acid for a few minutes to liberate free cystine, and then immersing in sodium naphthaquinone-4-sulphonate) shows a slight diffusion of stain, which is however again essentially confined to the cortex (Fig. 1*c*).

The Pauly reagent (diazotised sodium sulphanilate) not only distinguishes clearly the structural form of the medulla from the lamellae of the cortex, but indicates chemical differences as well.

This reagent is well known to give similar brown-red colorations with a number of the naturally-occurring amino-acids, but according to Totani [1915] when the stain originally developed is treated with zinc and hydrochloric acid, with subsequent addition of ammonia until alkaline, only the tyrosine and histidine stains remain, the former rose-red, the latter golden yellow. Moreover on oxidation of these secondary colorations with hydrogen peroxide, the tyrosine colour disappears leaving only a lemon-yellow histidine coloration,

[1] We are indebted to Dr C. Rimington for these analyses.

which is stable to this oxidising agent. Convenient practical details for these tests are given by Burgess and Rimington [1929].

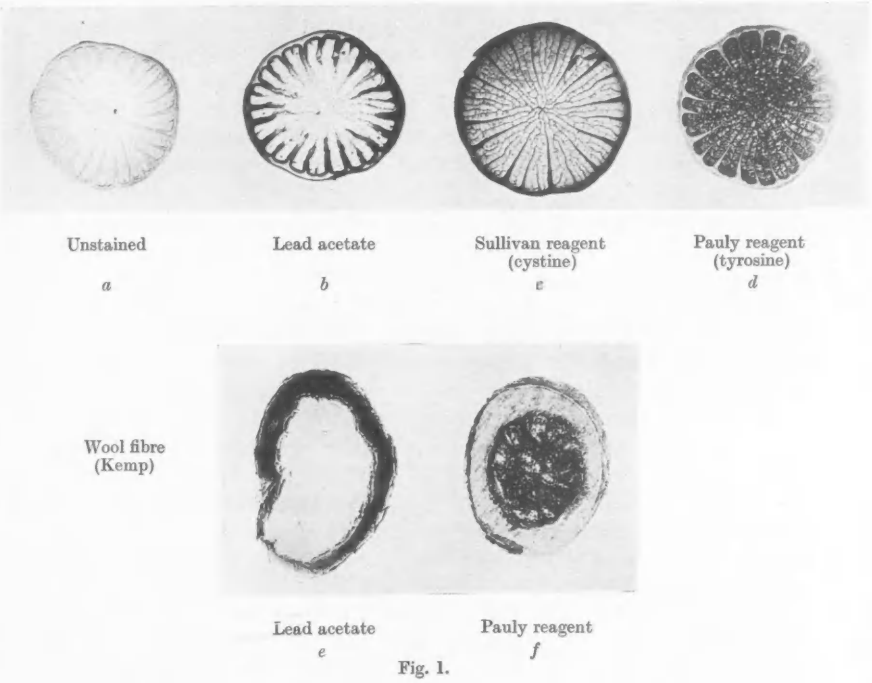

Fig. 1.

With sections of non-medullated wool fibres these colour changes are readily followed, the final lemon-yellow histidine stain being distributed throughout the cortex. Sections of medullated wool fibre however behave anomalously, in that the pronounced red-brown stain of the medulla is resistant both to the reducing and oxidising actions. Thus elimination of the tyrosine stain leaves a more brownish stain, equally persistent with the histidine stain remaining in the cortex, and masking any residual histidine stain which may also be given by the medulla (Fig. 1*f*).

Porcupine quills show similar behaviour, though the residual histidine stain of the cortex is very faint compared with that of wool fibre (Fig. 1*d*).

The photographs of course give a very inadequate impression of the striking colour differences.

Thus the chemical distinctions in the various components of wool fibre may be summarised:

Scales. Cystine, positive; tyrosine (and ? histidine), negative.
Cortex. Cystine, positive; tyrosine and histidine, positive.

Medulla. Cystine, negative; tyrosine (and ? histidine), positive; also specific reaction to Pauly reagent not shown by scale or cortex, indicating some constituent, as yet unidentified, peculiar to the medulla.

In contrast, picric acid, regarded as a specific keratin stain, produces no distinguishing effects, but a general stain for scale, cortex and medulla alike.

Thus the differential staining effects with both wool and quill sections confirm the view previously advanced that medulla cells are substantially devoid of sulphur, and that keratinisation, so far as scleroproteins such as wool keratin are concerned, is characteristically the result of incorporation of the cystine nucleus.

We therefore prefer to regard the term "keratinisation" as synonymous with cystinisation in contrast to general hardening or cornifying effects occurring in protein structures.

If we accept the histological view that scale, cortex, and medulla are all developed from the same primary plasmic material of the basal layer of the epidermis, the cornification process must be different in each case. That of the scale cells essentially involves true keratinisation or cystinisation, apparently without incorporation of the tyrosine nucleus; that of the cortex involves both cystine and tyrosine incorporation, while the hardening of the medulla cells, mainly a drying up of the semi-fluid contents to form hollow cells, involves in addition a certain degree of cornification associated with some keratinising agent (using the term in the general sense) other than cystine, the nature of which must await further chemico-histological study of the follicle and skin tissues.

In conclusion the authors express their thanks to Mr C. G. Winson, Miss Walker and Miss Hind for their valuable assistance with the photographic reproductions.

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CXXIII. VITAMIN C IN THE ORANGE AND THE GRAPE FRUIT.

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(*Received June 22nd, 1931.*)

Introduction.

The experiments described in this report are a part of a general investigation concerned with the bearing of the antiscorbutic factor on the biological activity of the plant. They have been carried out in a similar way to those made in the study of this vitamin in the apple [Bracewell, Hoyle and Zilva, 1930; Bracewell, Kidd, West and Zilva, 1931; Bracewell, Wallace and Zilva, 1931], with the aim of correlating the antiscorbutic activity of the fruit with its environment.

In this communication the influence of the season on the potency of the orange and of season and variety on that of the grape fruit has been investigated. It is evident that carefully controlled material which could be conveniently transported to meet the requirements of the enquiry was necessary and with this end in view Palestine was chosen as the source of the orange and British Honduras and South Africa as those of the grape fruit.

Concordant results were obtained during two consecutive seasons and indications are available that the time of picking has no influence on the activity of the juice of the orange or of the grape fruit, and in the case of the latter no very significant difference was observed in the potency of the juice of the several varieties investigated. There is some evidence, however, that those fruits, which yield a diminished amount of juice with the progress of the season and on storage, also yield a correspondingly smaller amount of vitamin since there is no change in the minimum protective dose of the juice. This would suggest that the antiscorbutic factor or one or more of the substances which contribute to its stability [Zilva, 1927, 1928, 1929, 1930] are utilised in the process of ageing of the fruit.

Technique.

The method of assay of vitamin C was the same as that employed in the previous investigation [Bracewell, Hoyle and Zilva, 1930]. The test was continued in most cases for 90 days. A prolonged test was important in these experiments since any serious deterioration in the antiscorbutic activity of a

[1] Member of the Scientific Staff of the Medical Research Council working with grants for assistance and expenses.

batch of fruit during a short period of storage would in all probability have been reflected in the weight curves.

The antiscorbutic activity of the Jaffa (Shamuti) orange.

Oranges are exported from Palestine from November to April inclusive; the bulk of the fruit is normally shipped in December, January, February and March. It was therefore desirable to examine samples collected at the beginning, middle and end of the season. This was done in the first experiment in 1928/29. During the following season material from the beginning and from the end of the season only was tested for confirmatory purposes.

Nine adjacent trees, planted at 5·5 metres distance and bearing 27 cases of fruit, in a grove situated 3 miles north of Jaffa, were set aside for the experiment. Fruit was picked from all the nine trees at the different periods of the season. The trees, about 25 years old, were healthy and were budded on bitter orange stock (*Citrus bigaradia*) in their second year. They were growing on fairly heavy loamy soil. The manurial treatment was as follows.

To each tree was added:

100 kg. old horse manure in October, 1927.
1·125 ,, sulphate of ammonia in March, 1928.
,, ,, ,, ,, June, 1928.
,, ,, ,, ,, August, 1928.
100 ,, old horse manure in October, 1928.

The same group of trees was used for the 1928/29 and 1929/30 experiments. Table I gives the dates of picking *etc.* In the 1929/30 season the oranges were

Table I.

Batch	Picked	Beginning of biological test
V 1, 1928	Early November	11. xii. 28
V 4, 1929	Early January	5. ii. 29
V 9, 1929	Early March	15. iv. 29
V 1, 1929	Early December	2. i. 30
V 9, 1930	Early March	2. iv. 30

exceptionally late in maturing and consequently the first batch was picked about 3 weeks later than the corresponding batch of the previous year.

The tests for vitamin C were begun immediately on the arrival of the fruit. The oranges were stored at a temperature of about 15° during the three months' duration of the test. The juice was expressed daily before dosing and the quantity yielded by each fruit noted.

The chemical constitution of orange juice changes when the fruit is held on the tree and when kept in storage. During the process of ripening the soluble solids content of the juice increases, especially during the last 2 or 3 weeks [Copeman, 1931]. There are also a gradual increase of total sugar, sucrose and reducing sugars increasing at about the same rate, and a decrease of acidity as the oranges mature [Collinson, 1913].

Circumstances did not permit in this investigation of many chemical analyses being made, but the changes observed were broadly in agreement with those previously recorded by workers who had studied this aspect of the subject in greater detail.

Table II gives a 10-day average of the yield of juice per orange of the

Table II. *Daily average yield of juice per orange. The duration of the test is divided into 10-day periods.*

Period	V 1, 1928 cc.	V 4, 1929 cc.	V 9, 1929 cc.	V 1, 1929 cc.	V 9, 1930. A cc.	V 9, 1930. B cc.
1	67·60	65·70	42·46*	66·66	53·33	63·33
2	65·40	57·24*	50·75	69·00	48·50	65·50
3	63·70	68·29*	43·78	67·25	50·00	70·00
4	79·10*	60·43	41·50	61·30*	45·00*	60·00*
5	58·88*	54·62	41·11	65·33	53·01	58·33*
6	63·55	62·17	35·50	58·00	35·00	51·66
7	43·14	61·50	32·11	50·00	—	51·33
8	43·90	58·87	20·52	45·10	—	48·30

* Denotes period during which the juice was analysed. Results are given in Table III.

various batches as the tests proceeded. It is seen that the yield decreases as the test of each batch proceeds. It decreases further as the season advances. The last batch (V 9) of the 1929/30 season arrived in two different containers, A and B, and the oranges from each box were removed on alternate days for the tests so as to obtain an average result. It will be seen, however, that there was a marked difference in yield of juice by the fruit of the two consignments,

Table III.

Batch	Date of estimation	Total solids g./100 cc.	Acid in g. Citric acid/ 100 cc.	Maturity ratio	p_H of juice
V 1, 1928 Early November	12. i. 29	9·49	1·35	7·1	3·06
		8·72	1·29	6·8	3·11
		8·11	0·68	12·0	3·62
	25. i. 29	9·39	1·32	7·2	3·13
		9·43	1·27	7·4	3·23
V 4, 1929 Early January	19. ii. 29	10·93	0·94	11·6	3·29
		12·00	0·98	12·3	3·35
		9·86	1·37	7·2	3·23
	6. iii. 29	9·32	0·94	10·0	3·33
		9·83	1·10	8·9	3·23
V 9, 1929 Early March	15. iv. 29	12·50	0·73	17·0	3·75
		11·89	0·77	15·4	3·64
		12·81	0·46	27·7	3·95
	18. iv. 29	10·40	0·62	16·9	3·72
		11·55	0·33	35·4	4·20
V 1, 1929 Early December	12. ii. 30	10·42	0·79	13·15	3·02
		8·70	0·93	9·34	2·87
		9·02	0·79	11·58	2·99
V 9, A 1930	13. v. 30	10·57	1·17	9·06	3·45
		11·24	0·85	13·32	3·60
B 1930 Early March	9. v. 30	10·21	0·56	15·66	3·90
		11·89	0·79	15·02	3·72
	18. v. 30	10·61	0·95	11·23	3·48
		10·76	1·02	10·50	3·72

although they were picked at the same time and in the same grove. The average yield of the two is lower than that from the first batch, V 1, of this season.

Table III sets out the analytical data of the various batches. Unfortunately, as will be seen from Table II, the analyses could not be carried out in corresponding periods of the testing time. Nevertheless, they give a fair survey of the changes that took place in the composition of the juice with the progress of the season. It will be seen that in the 1928/29 season there was an increase in the soluble solids as the oranges remained longer on the tree. The acid content and the hydrogen ion concentration decreased. The maturity ratio (ratio of soluble solids to acidity calculated as citric acid) rose in consequence as the season advanced. The figures for the season 1929/30 did not show quite the same trend, but this is possibly due to the abnormality of the season.

The vitamin C assays (Table IV) do not reveal any marked difference in

Table IV. *Antiscorbutic potency of juice from Jaffa oranges.*

Dose cc.	V 1, 1928 Picked early November	V 4, 1929 Picked early January	V 9, 1929 Picked early March	V 1, 1929 Picked early December	V 9, 1930 Picked early March
½	No protection	No protection	?	+	±
1	+ + +	+ + +	+ + + ±	+ + + ±	+ + + ±
1½	Not tested	+ + + ±	+ + + +	+ + + +	+ + + +
2	+ + + +	+ + + +	+ + + +	Not tested	Not tested
3	+ + + +	Not tested	Not tested	+ + + +	+ + + +

potency within the limits of the gradation of the tested doses. There was possibly a slightly higher potency in the juices of V 9, 1929 and V 1 and V 9, 1930. This disparity is, however, of such a low order that it cannot be considered significant. The appearance of the weight curves was normal and the *post mortem* findings were consistent, which suggests that a significant loss in the antiscorbutic potency was not incurred during at least the first two months of the test.

The antiscorbutic activity of grape fruit from British Honduras.

The export season in British Honduras lasts from October to the beginning of April, and samples were examined which were collected at the beginning, the middle and the end of the season during the first year and at the beginning and at the end of the season during the second year. Two varieties, namely Duncan and Marsh, were investigated. Owing to a misunderstanding the two varieties were mixed in the first season's tests. The following year however, each variety was tested separately in the autumn in order to ascertain whether they differed in potency.

The fruit was collected from two healthy trees from a grove in British Honduras situated 14 miles from the sea coast on the lower slope of a hill at an altitude of about 100 ft. above sea level. The trees were imported from the United States and were planted in the present grove in 1914. The age of

the trees and nature of the stock is not known. The tree from which the Duncan variety was picked is grown in 18 in. of reddish-brown, alluvial silt grading to a dry, dense bright red micaceous clay subsoil, p_H 6·9. The Marsh variety is grown on 14 in. of reddish brown, alluvial silt grading to a reddish yellow, gritty and micaceous dry clay subsoil, p_H 6·9. Grass grows throughout the grove which is grazed by mules. Grazing has caused the surface to become somewhat packed. The trees were sprayed with Bordeaux sulpho-citrol four times a year. 15 lb. per tree of a manurial mixture containing 10 % of phosphoric acid, 6 % nitrogen and 7 % of soluble potash was forked in during March and April.

The vitamin tests were begun immediately on the arrival of the fruit, which was stored at about 15°. The juice was expressed daily, but owing to the size of the fruit only a part of it was used. The remainder was kept in cold store at 0° until the fruit was finished, usually 3 days. Table V gives dates of picking and the beginning of the tests.

Table V.

Batch	Picked	Beginning of biological test
G 1, 1928. Marsh and Duncan	Early December	14. i. 29
G 3, 1929. Marsh and Duncan	Early January	20. iii. 29
G 4, 1929. Marsh and Duncan	Early March	10. iv. 29
G 1, 1929. Marsh	Early October	12. xi. 29
G 1, 1929. Duncan	Early October	13. xi. 29
G 4, 1930. Marsh	Early April	13. v. 30

The changes which take place in the chemical constitution of the grape fruit juice are briefly the following. Collinson [1913] found a decrease in acidity and an increase in the sugar content as the season advanced. After the fruit has matured the sucrose is gradually hydrolysed to reducing sugar. Zoller [1918] observed a decrease of acid content during storage and an increase of sugar after the removal of the fruit from the tree. According to Hawkins and Magness [1920] the acid content decreases and there is an apparent increase in sugar during cold storage. The dry matter increases during storage. There is a shrinkage from 5–8 % in cold storage to 25 % in warm ventilated storage. Hawkins [1921] found further that in warm storage there is a marked increase in the percentage of the acid calculated on the wet weight of the pulp, which is entirely due to loss of water. There is also possibly a slight decrease in the sugar content. In cold storage the acidity decreases markedly after 4 months whilst there is little change in the sugar content.

Table VI presents the analytical data obtained from the fruit used in this investigation. No very marked difference between the various periods can be discerned from these few data. The figures, however, suggest that the soluble solids and the acid content of the Duncan variety of 1929/30 season were lower than those of the Marsh variety of the same season.

The antiscorbutic potency of the grape fruit is summarised in Table VII. The general evidence points to the fact that this fruit, too, does not vary

Table VI.

Batch	Date of estimation	Total solids g./100 cc.	Acid in g. citric acid/ 100 cc.	p_H of juice
G 1, 1928	28. ii. 29	9·76	1·03	3·16
Marsh and Duncan		10·43	0·58	3·65
Early December	4. iii. 29	11·45	0·74	3·23
		10·83	0·76	3·30
G 3, 1929	7. iii. 29	10·53	0·88	3·32
Marsh and Duncan		12·97	1·18	3·18
Early January	23. iii. 29	10·12	0·75	3·43
		10·27	0·66	3·51
G 4, 1929	10. iv. 29	9·89	0·87	3·35
Marsh and Duncan		9·97	0·62	3·62
Early March	16. iv. 29	11·34	0·73	3·48
		11·61	0·76	3·44
G 1, 1929	8. i. 30	10·24	1·17	3·29
Marsh		10·56	1·10	3·20
Early October				
G 1, 1929	8. i. 30	8·90	0·89	3·16
Duncan		8·81	0·69	3·31
Early October				
G 4, 1930	16. vi. 30	10·08	0·95	3·25
Marsh		10·45	1·00	3·38
Early April		10·26	1·09	3·29

Table VII. *Antiscorbutic potency of juice from grape fruit. British Honduras.*

Dose cc.	G 1, 1928 Marsh and Duncan Picked early December	G 3, 1929 Marsh and Duncan Picked early January	G 4, 1929 Marsh and Duncan Picked early March	G 1, 1929 Marsh Picked early October	G 1, 1929 Duncan Picked early October	G 4, 1930 Marsh Picked early April
½	+ −	?	?	+ +	+ + +	+ −
1	+ + + + ?	+ + +	+ + +	+ + + +	+ + + +	+ + + + ?
1½	Not tested	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
2	+ + + +	+ + + +	+ + + +	Not tested	Not tested	Not tested
3	+ + + +	Not tested	Not tested	Not tested	Not tested	+ + + +

within the limits of the dosage used in these tests, with the time of season. Nor do the weight curves reveal a very marked loss in activity during the first 2 months of the test period. The results call, however, for some comment. The mixed juices of the Marsh and Duncan of December 1928 showed a somewhat higher vitamin content than did those of January and of March 1929. The difference in activity is of an order which falls outside the limits of the experimental error. It is difficult, however, to accept the view that this falling off in activity was due to the longer sojourn of the fruit on the tree, since no significant difference was observed between the activity of the Marsh variety picked in October and that of this fruit picked in April of the following season.

A somewhat marked falling off in weight of the guinea-pigs on the 1 cc. dose of the Marsh picked in April 1930 was observed towards the end of the test. This could hardly be due to the deterioration of the activity of the fruit during the test period, since the manifestation of scorbutic symptoms should

have preceded the decline in weight if it were due to scurvy. This did not occur except in one animal. Nor did the *post mortem* examination of the animals suggest that the loss in weight was due to scurvy.

It must be noted that the Duncan variety, in spite of lower total solids, showed a somewhat higher vitamin content than the Marsh variety.

THE ANTISCORBUTIC ACTIVITY OF GRAPE FRUIT FROM SOUTH AFRICA.

These experiments were instituted in order to ascertain whether there is a difference in the antiscorbutic potency of the same variety (Marsh) emanating from a different environment. An additional variety (Florida) was also assayed.

The South African grape fruit season lasts from the middle of May to the middle of August. The two varieties were received early in September.

Table VIII. *Antiscorbutic potency of juice from grape fruit. South Africa.*

Dose (cc.)	1930. Marsh No protection	1930. Florida
$\frac{1}{2}$		−
1	+ + + −	+ + + −
$1\frac{1}{2}$	+ + + +	+ + + +
3	+ + + +	+ + + +

The Marsh variety was grown at Simondium, Western Cape, on Karroo silt. The trees were 12 years old, healthy and budded on Rough Cape Lemons. They were planted in the grove in 1918. They were previously bad with scale, but are fairly clear now and were not sprayed. The soil was ploughed annually and has been irrigated six times annually since March 1928. Previous to this, owing to drought, irrigation was erratic, the trees going sometimes for as long as six months without water. The irrigation was followed by cultivation. They have been fertilised each autumn with kraal manure since 1926.

The Florida variety was grown in the Eastern Cape Province on Karroo silt. The trees were 8 years old, healthy and budded on Rough Cape Lemons. They were planted in 1922 and were not sprayed. The method of irrigation, cultivation and manuring was the same as for the Marsh variety.

As will be seen from Table IX, these batches of grape fruit had a higher

Table IX.

Batch	Date of estimation	Total solids g./100 cc.	Acid in g. citric acid/100 cc.	p_H of juice
1930. Marsh	2. x. 30	10·31	1·44	2·71
	14. x. 30	9·67	1·46	2·80
	17. x. 30	10·18	1·78	2·83
1930. Florida	2. x. 30	8·69	1·69	2·83
	14. x. 30	8·51	1·78	2·87
	17. x. 30	8·26	1·76	2·71

acid content and lower p_H value than the British Honduras varieties. The Florida variety showed a total solids content similar to that of the Duncan variety from British Honduras. The vitamin content (Table VIII) was of the

same order as that of the corresponding British Honduras Marsh variety. It is true that the South African Marsh variety shows slightly lower activity than the one of British Honduras origin, but the difference is too small to be considered significant under the circumstances.

Conclusions.

These systematic observations on the constant antiscorbutic activity of the orange and grape fruit during the entire season and on the stability of the vitamin in the intact fruit are consistent with the scattered observations made by various workers in this connection. The vitamin C activity of the orange, lemon and grape fruit has very seldom been found to vary to any great extent and evidence is also available concerning its stability. Thus Davey [1921] observed that 5 months of cold storage did not appreciably impair the vitamin C content of the orange. The activity of the orange, as we have seen from the foregoing experiments, like that of the grape fruit, does not seem to fluctuate markedly with the variety. The minimum dose necessary to protect the guinea pig from scurvy, as found in this investigation, is about the same as that recorded by Delf [1921] and Davey [1921] for Denia and Navel oranges. Taking all the available information into consideration one may safely assume that oranges and grape fruit bought casually in the open market under normal conditions ought to possess the full antiscorbutic value characteristic of these fruits.

A point of academic interest which requires further investigation is whether the total amount of vitamin of the Citrus fruits diminishes as they age on the trees or in storage. The yield of juice of the Jaffa orange diminished with the progress of the season when the fruit was left on the tree and during the 3 months' storage in the course of carrying out the test. The antiscorbutic potency of the juice on the other hand did not show an increase. The difference in the yield of the orange juice between the first and last pickings of the 1928/29 season ranged from 25 to 53 % for the corresponding periods, the average being 35 % (Table II). A similar increase in the potency in the juice should have been reflected in the test. A closer spacing of the doses would most probably establish definitely whether or not such an increase takes place.

The small disparity in the vitamin C content of the orange, grape fruit, lemon and sweet lime (*Citrus limetta*) as contrasted with the West Indies lime (*Citrus medica* var. *acida*), which possesses a definitely lower antiscorbutic activity than the aforementioned Citrus fruits, presents an analogy to the variation in the antiscorbutic activity amongst the apple varieties. In the apples, the antiscorbutic activity of which is on a lower plane than that of the Citrus fruits, the Bramley's Seedling, and, according to evidence which is now forthcoming, also some other varieties are markedly more potent than another category of apples such as Cox's Orange Pippin, Worcester Pearmain *etc.*, which differ amongst themselves comparatively little [Bracewell, Hoyle

and Zilva, 1930]. This sharp division in antiscorbutic activity amongst closely related fruits is rather striking and calls for further attention.

Summary.

1. The antiscorbutic activity has been determined of (*a*) the Jaffa orange picked from the same trees at different times of the season during two consecutive years, (*b*) of the Marsh and Duncan varieties (British Honduras) of grape fruit also picked at different times of the season during two consecutive years and (*c*) the Marsh and Florida varieties (South Africa) of grape fruit picked at the end of the season.

2. Within the limits of the doses tested and of the accuracy of the method employed, the vitamin content of the juice is the same whether the orange or grape fruit is picked at the beginning or at the end of the season.

3. There is no marked loss in the vitamin content when the fruit is stored under ordinary conditions at about 15° for about 2 months.

4. The conditions of cultivation, origin of stock, age of tree, soil *etc.* do not affect the vitamin C content of the fruit.

5. The Marsh variety of grape fruit whether of British Honduras or of South African origin, is of approximately the same potency.

6. The Duncan variety of grape fruit (one test only) was found to be slightly more active than the other two varieties of grape fruit tested.

7. The vitamin C content of the grape fruit was rather higher than that of the Jaffa orange.

8. There is no correlation between the soluble solids, acid content, p_H of the juice and its antiscorbutic potency.

We should like to take this opportunity of acknowledging our indebtedness to Mr A. C. Shill, of the Empire Marketing Board, and Mr S. T. Tolkowsky, for collecting and supplying the details for the Jaffa oranges, to Mr H. P. Smart, Agricultural Officer at British Honduras, and to both Mr J. Dimond, Trade Commissioner for South Africa, and Mr Harlow, of the South African Co-operative Citrus Exchange, Ltd., for similar help with the grape fruit. Our thanks are also due to Mrs M. A. Boas-Fixsen for helping us with some of the biological tests.

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CXXIV. THE CHLORIDES AND INORGANIC CONSTITUENTS OF THE SERUM AND CEREBROSPINAL FLUID IN NEPHRITIS AND ACIDOSIS.

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In view of the constant relationship of the chlorides of the cerebrospinal fluid to the chlorides of the blood-serum in normal conditions [Hamilton, 1925] and in meningitis [Linder and Carmichael, 1928], it appeared to us of interest to investigate the conditions which obtain in nephritis and in ammonium chloride acidosis. The few observations which we have found in the papers of Hamilton and of Pincus and Kramer [1923] have not revealed any striking changes.

It has been reported [Greenfield and Carmichael, 1925] that in uraemia the cerebrospinal fluid chloride may rise from the normal level of 720–745 mg./100 cc. to 900 or even 1000 mg./100 cc. We have found no record of serum analyses in these cases, but, if we assume the normal relationship to hold, the serum-chloride corresponding to these figures would be 745 and 830 mg. per 100 cc. of serum (127 and 142 *mM*). That this is the case with any but the lesser increases seems highly improbable, for Bulger, Peters, Eisenman and Lee [1926] in 77 cases of nephritis found 5 with a serum-chloride exceeding 100 *mM* and none exceeding 128 *mM*, and Atchley and Benedict [1930] found that a decrease in serum-chlorides and total serum-electrolytes was the rule in uraemic acidosis. No patient presenting such gross increase in cerebrospinal fluid chloride has been available for us to study, but the suggestion that the usual relationship of cerebrospinal to serum-chlorides is abrogated was supported by one of our observations.

Experimental.

Cerebrospinal fluid and arterial blood-serum were obtained and investigated by the methods described in our earlier paper [1928] with the exceptions that the inorganic phosphorus was determined by the method of Fiske and Subbarow [1925], and that the water content of the serum was calculated from a serum-protein determination by the formula, water = (990 − 0·8 × protein) g. per litre [Van Slyke, Wu and McLean, 1923]. As before the concentration of the inorganic constituents was recorded in *mM* concentration in serum-water and in cerebrospinal fluid.

Table I.

Case no.	Diagnosis	Blood urea (mg./100 cc.)	Blood pressure (mm. Hg)	Chloride: Serum-water (mM)	Chloride: CSF (mM)	Cl_{sw}/Cl_{csf}	Bicarbonate: Serum-water (mM)	Bicarbonate: CSF (mM)	$BHCO_{3sw}/BHCO_{3csf}$	Total base: Serum-water (mM)	Total base: CSF (mM)	B_{csf}/B_{sw}	Inorganic phosphorus: Serum-water (mM)	Inorganic phosphorus: CSF (mM)	Protein: Serum (g./100 cc.)	Protein: CSF (g./100 cc.)	Other acids: Serum-water (mM)	Other acids: CSF (mM)	p_H: Serum	p_H: CSF	Remarks
I	Hydraemic nephritis	116	125/60	119	129	0·92	18·8	20·7	0·91	171	162	0·95	2·0	1·1	3·5		22	11	7·38	7·41	Urea therapy
II	Azotaemic nephritis	95	265/160	98	116	0·85	33·5	28·65	1·17	170	161	0·95	1·8	0·7	8·1	0·02	17	15	7·52	7·37	Alkali therapy
III	Azotaemic nephritis, uraemia	200	180/120	118	130	0·91	12·5	17·8	0·70	164	152	0·93	2·1	0·3	7·9	0·03	13	4	7·23		
IV	Azotaemic nephritis, uraemia	380	240/120	98	127	0·77	13·0	13·2	0·98	164	(133)		4·7	5	7·0	0·09	26		7·26		
	Mean normal ...	...	...	111	124	0·89	25	21	1·19	164	156	0·95	1·2	0·6	—	—	—	—	—	—	Hamilton's series

Table II.

Exp.	Chloride: Serum-water (mM)	Chloride: CSF (mM)	Cl_{sw}/Cl_{csf}	Bicarbonate: Serum-water (mM)	Bicarbonate: CSF (mM)	$BHCO_{3sw}/BHCO_{3csf}$	Total base: Serum-water (mM)	Total base: CSF (mM)	B_{csf}/B_{sw}	Inorganic phosphorus: Serum-water (mM)	Inorganic phosphorus: CSF (mM)	Protein: Serum (g./100 cc.)	Protein: CSF (g./100 cc.)	Other acids: Serum-water (mM)	Other acids: CSF (mM)	p_H: Serum	p_H: CSF	Remarks
I a	105	122	0·86	32	27·6	1·16	157	154	0·97	1·5	0·55	6	0·09	3	3	7·45	7·44	Before NH_4Cl
b	117	127	0·92	20·1	23·4	0·86	154	158	1·03	1·55	0·6			− 1	7	7·35	7·38	After 2 days on 12 g./day
c	111	127	0·88	20·15	22·2	0·91	155	154	0·99	1·18	0·47	6·5		1	4·5	7·41	7·40	After 4 days on 12 g./day
II a	107	120	0·89	30·5	26·7	1·14	160	150	0·92	1·18	0·48	7	0·05	2	2	7·52		Before NH_4Cl
b	114	123·5	0·92	22·7	22·8	1·00	161	151	0·94	0·95	0·47	9	0·04	3	5	7·43		After 2 days on 12 g./day

Nephritis.

Table I presents the results obtained in four cases of nephritis. The mean of Hamilton's series is given as a guide to the normal.

Chloride. In one patient, Case II, the cerebrospinal fluid chloride was less than normal. The serum-chloride was also low and so the ratio of Cl_{sw} to Cl_{csf} was the same as in the normal series. The bicarbonate was rather high, and both of these abnormalities were attributed to the fact that until the preceding day the patient had been taking large doses of alkali.

In the remaining patients the cerebrospinal fluid chloride was just above the normal range. In two, Cases I and III, there was a proportionate increase in the serum-water chloride, but in the third, Case IV, the cerebrospinal fluid chloride was high whereas the serum-water chloride was low, and the ratio Cl_{sw}/Cl_{csf} fell to the very unusual figure of 0·77. Such a low ratio appears to be exceptional. Among Hamilton's observations there were two as low as 0·83, one from a case of cerebral tumour and the other from a case of uraemia. In the latter the cerebrospinal fluid chloride was high, 142 *mM* (830 mg./100 cc.); the serum-bicarbonate was 15 *mM*, so there was a moderately severe acidosis. In our own case there was advanced acidosis, the serum-bicarbonate being 12·1 *mM*, the p_H 7·26 and the inorganic phosphorus 4·4 *mM* (13·6 mg./100 cc.).

Bicarbonate. The serum-bicarbonate was very low in Cases III and IV, and was below normal in Case I; in the remaining patient it was high, probably as a result of alkali therapy.

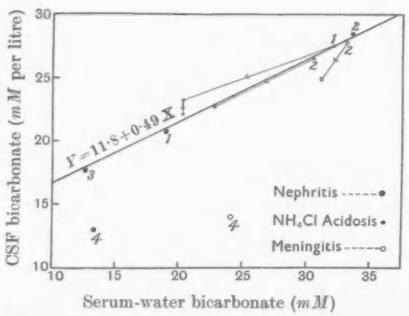

Chart showing relation of serum-water bicarbonate to cerebrospinal fluid bicarbonate. The numbers correspond to the case numbers. The points joined by thin lines are from the same case and the arrows indicate the order in which the points are to be read. Two meningitis cases with very high bicarbonate are omitted.

Hamilton found that the serum-water bicarbonate (X) showed a relationship to the cerebrospinal fluid bicarbonate (Y), which was expressed best for his series by the equation $Y = 9{\cdot}7 + 0{\cdot}46\,X$, X and Y being measured in *mM*. We have plotted the points for our observations in meningitis [1928], nephritis and ammonium chloride acidosis, and found that they tended to lie along a line parallel to but a little higher than Hamilton's; our line corresponded

to the equation $Y = 11{\cdot}8 + 0{\cdot}49\,X$. Three points out of 14 lay far away from this line; two were from meningitic patients, one having a very high and the other a very low serum-bicarbonate, and the third was Case IV of this paper, who had severe acidosis and the abnormal chloride ratio commented upon above.

It is noteworthy that in Case III a still greater degree of acidosis was present, but both the cerebrospinal fluid chloride and bicarbonate were at the levels expected from the normal chloride ratio and the bicarbonate equation.

Total Base. There was no abnormality of total base in these cases, but in Case IV the determination in the cerebrospinal fluid was obviously erroneous and unfortunately could not be repeated.

Ammonium chloride acidosis.

Table II gives the results of producing an acidosis with ammonium chloride in two patients with quiescent tabes. Twelve grams of ammonium chloride were given daily. Cerebrospinal fluid and arterial blood were taken before and after two and four days' treatment. The results were a reduction of serum-bicarbonate by a third, a moderate fall in p_H, and a rise in serum-chloride and serum-protein. The cerebrospinal fluid chloride followed the serum-chloride with possibly a slight delay; the ratio Cl_{sw}/Cl_{csf} became slighly greater, but the normal balance was maintained. The bicarbonate figures followed the line discussed above very closely. The total base ratio was not disturbed.

Summary and conclusions.

It is apparent from these figures that in moderate disturbances of acid-base balance due to nephritis or to ammonium chloride therapy the normal relationships of the cerebrospinal fluid and arterial blood-serum remain intact.

In severe nephritis with great reduction in serum-bicarbonate and p_H and gross nitrogen retention a breakdown of these relationships may sometimes occur as in Case IV and in Hamilton's case McG-n, in which the cerebrospinal fluid chloride was considerably higher than was compatible with the serum-chloride. In Case IV, too, the cerebrospinal fluid bicarbonate was considerably less than was to be expected from the serum-bicarbonate, and the inorganic phosphorus in the cerebrospinal fluid was vastly increased and practically equal to that in the serum. We are of the opinion that these observations give the key to the situation in those cases of uraemia in which very high cerebrospinal fluid chlorides have been reported and explain why such high cerebrospinal fluid chlorides are of evil prognosis.

We wish to express our gratitude to Prof. F. R. Fraser, Prof. A. W. Falconer, and Dr George Graham for permitting us to make observations on their patients, and to the Medical Research Council for personal grants received during part of the time this work was in progress.

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CXXV. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

VIII. THE METABOLISM OF GLUTATHIONE COMPARED WITH THAT OF OTHER CYSTINE DERIVATIVES.

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THE remarkable activity of glutathione *in vitro* and its wide distribution in different animal tissues have, in recent years, focussed attention on its metabolic function. So far however no wholly satisfactory explanation of the high concentration of glutathione in such diverse tissues as liver, lens, and testis has been put forward. The possibility therefore suggests itself that it is a metabolic end-product and that its occurrence is due to an animal's inability to decompose it. A comparison of the behaviour of glutathione and cystine when administered to a dog should throw light on this question since the latter substance is only present in traces, if at all, in such fresh, non-pathological, tissues as have been studied. Some difference in the degree of oxidation was to be expected since Abderhalden and Samuely [1905] have found that less than half of the sulphur given to a dog as dialanylcystine or dileucylcystine is excreted as sulphate. We find however that there is no difference between the behaviour of glutathione and that of cystine in the dog. Of all the cystine-containing peptides which have been studied glutathione is the most completely oxidised[1].

EXPERIMENTAL.

The methods employed in this work were identical with those already described in the earlier papers of this series [Hele, 1924]. Three bitches were used and they received the following diets, half being given in the morning and half in the afternoon. They were catheterised at the same hour each morning and fed immediately afterwards.

	Lean meat	Sugar	Margarine	Milk	Water
	g.	g.	g.	cc.	cc.
Perky (10·5 kg.)	80	70	20	160	100
Violet (10·5 kg.)	80	70	20	160	100
Patsy (8·2 kg.)	80	50	20	160	100

When given orally the substances studied were put in small gelatin capsules which were wrapped up in a thin slice of meat (part of the diet) and when injected they were dissolved in 5–10 cc. of water containing, in the case of acids, the amount of sodium hydroxide necessary to bring the p_H to about 7.

[1] It has been shown in some unpublished work by Hele, Hopkins, Lawrie, Leese, Meldrum and Pirie that a cystinuric could oxidise 3 g. of glutathione, given by mouth, as readily as cystine.

On two of the dogs we tested the experimental technique by injecting subcutaneously 3·22 g. of sodium sulphate (decahydrate) dissolved in 10 cc. of water. The "extra" sulphate in the urine on the following day was 98 % of the dose in each case.

Cysteine hydrochloride and *l*-cystine were prepared from human hair in the usual way and *r*-cystine was prepared from the latter by the method of Hoffman and Gortner [1922]. *r*-Cysteine hydrochloride was made by reducing the *r*-cystine with tin and hydrochloric acid. The methods of preparation of acetylcysteine, chloroacetylcysteine, dichloroacetylcystine, glycylcysteine and oxidised glutathione have already been described [Pirie, 1931] as has the method of preparation of reduced glutathione [Pirie, 1930].

These substances are not wholly without ill effect on the dogs. Unless the dose is fed in two portions some hours apart the acidic compounds occasionally cause vomiting and on the occasion when oxidised glutatione was injected the dog had a mild attack of diarrhoea. Experiments involving any alimentary disturbance have been excluded from Table I except in the case of oxidised glutathione given subcutaneously.

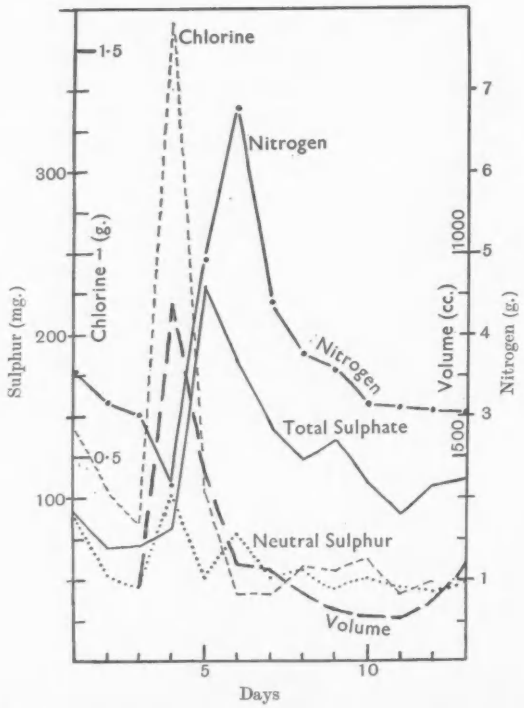

Fig. 1. Perky: 0·16 g. of glycylcysteine anhydride given subcutaneously on day 3. 0·5 g. of glycylglycine anhydride given subcutaneously on day 10.

Once the feeding of 1·2 g. of cystine on a low protein diet (*i.e.* meat 10 g., sugar 60 g., margarine 40 g., milk 50 cc., water 100 cc.) caused haematuria, but this did not happen on four other occasions although there was always a great diminution in the volume of urine passed on the day after the dose.

We attempted to study the metabolism of glycylcysteine anhydride prepared by the method of Hopkins [1929]. 1·2 g. was fed to Patsy on December 9th, she had a marked diuresis on the 11th, 12th, 13th and 14th; the daily volume of urine increased from about 210 cc. to 420 cc. and, since her fluid intake remained constant, she lost 600 g. weight. Her nitrogen and sulphur excretions were very erratic. In an experiment on Perky (Fig. 1) 0·16 g. of glycylcysteine anhydride was given subcutaneously on the 3rd day. The diuretic action and general metabolic upset are clearly shown in the graph. In the urine there were blood cells but no casts and the delayed excretion of

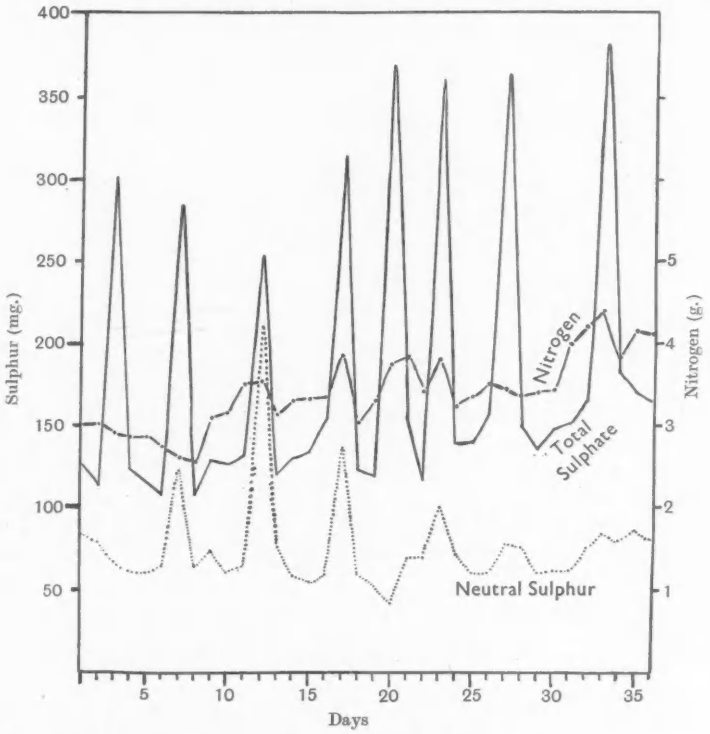

Fig. 2. Patsy: day 3. 1/100 g. mol. cysteine hydrochloride *sub cutem.*
7. 1/100 glycylcysteine *sub cutem.*
12. 1/100 acetylcysteine *sub cutem.*
17. 1/200 oxidised gluthathione *sub cutem* (see note, Table I).
20. 1/200 ,, ,, *per os.*
23. 1/100 *r*-cysteine hydrochloride *per os.*
27. 1/100 glycyl cysteine *per os.*
33. 1/200 cystine *per os.*

nitrogen and sulphur shows that there was some alteration in the permeability of the kidney. On the 10th day she received 0·5 g. of glycylglycine anhydride subcutaneously without any such effect. The oral administration of 0·3 g. of diglycylcystine dianhydride had a similar, but smaller, diuretic action while 1·2 g. of glycylglycine anhydride had none. Four rats, which had each received 6 mg. of glycylcysteine anhydride, all showed a similar diuresis, but even 0·1 g. was without effect on each of three rabbits whether they were fed on a diet of cabbage or on one of bread and milk. Lewis and Lewis [1927] found that dialanylcystine dianhydride was absorbed by a rat and Brand and Sandberg [1926] injected 70 mg. into a rabbit. In neither paper is any ill effect mentioned. Abderhalden and Wacker [1908] fed 10 g. of glycylglycine anhydride

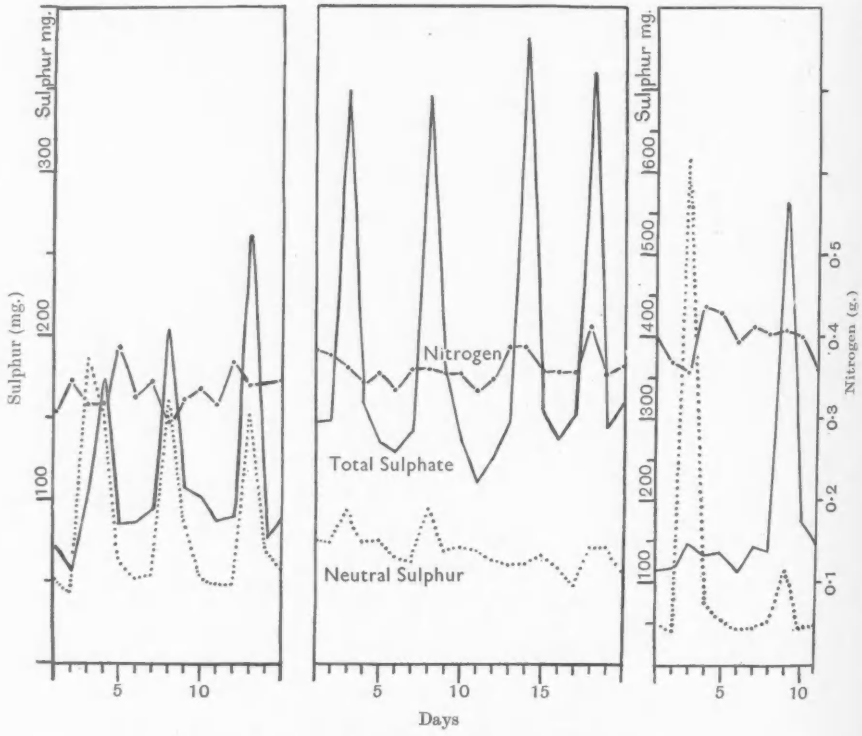

Fig. 3. Violet: day 3. 1/200 gm. mol. dichloroacetylcystine *per os.*
8. 1/100 acetylcysteine *per os.*
13. 1/100 *r*-cysteine hydrochloride *sub cutem.*

3. 1/100 cystine *per os.*
8. 1/100 *r*-cystine *per os.*
14. 1/100 reduced glutathione *per os.*
18. 1/100 ,, ,, *sub cutem.*

3. 1/100 dichloroacetylcystine *sub cutem.*
9. 2/100 cysteine hydrochloride *sub cutem.*

to a rabbit and mention a diuretic effect; they also found that most of it was absorbed. It is clear however that the diuresis caused by glycylcysteine anhydride is of an entirely different order from that due to glycylglycine anhydride. In spite of its fairly high solubility only a small part of the glycylcysteine anhydride which we fed to a dog seemed to be absorbed, and only 140 mg. (12 % of the dose) could be recovered from Patsy's urine although such a compound is relatively easy to isolate. This amount corresponded to the rise in neutral sulphur.

Only one experiment was carried out with *p*-iodophenylcysteine [Baumann and Schmitz, 1895] since 1·6 g. given orally made the dog vomit, and iodine estimations on the urine, vomit and faeces showed that less than 5 % of the dose had been absorbed.

Analytical data for some of the experiments are shown graphically in Figs. 2 and 3. In all cases there were two or three more blank days at the beginning and end of each set of experiments than are shown. The course of the experiments of which the results only are given was similar.

Table I.

Substance	Dog	Mg. S given	How given	% excreted as sulphate: 1st day	2nd day	Total	% excreted as neutral sulphur: 1st day	2nd day	Total	% accounted for
l-Cystine	P	320	Orally	66	7	73	0	0	0	73
,,	P.	160	,,	74	0	74	0	0	0	74
,,	V.	320	,,	70	0	70	7	0	7	77
,,	V.	320	,,	61	14	75	0	0	0	75
,, *	P.	320	,,	31	31	62	4	7	11	73
,, *	P.	320	,,	69	6	75	6	0	6	81
,, *	V.	320	,,	41	22	63	—	—	—	—
,, *	V.	640	,,	48	24	72	—	—	—	—
r-Cystine	V.	320	,,	67	13	80	9	0	9	89
d-Cysteine-HCl	V.	640	Subcutaneously	66	4	70	10	0	10	80
,,	P.	320	,,	58	3	61	0	0	0	61
,, †	V.	320	Orally	69	3	72	0	0	0	72
r-Cysteine-HCl	V.	320	Subcutaneously	54	0	54	31	4	35	89
,,	P.	320	Orally	72	0	72	10	0	10	82
,,	Pk.	320	,,	56	4	60	19	0	19	79
,, †	V.	320	,,	57	7	64	8	0	0	72
Acetylcysteine	P.	320	Subcutaneously	40	0	40	46	5	51	91
,,	V.	320	Orally	34	4	38	33	10	43	81
,,	Pk.	320	,,	50	0	50	28	0	28	78
Dichloroacetylcystine	V.	320	,,	14	26	40	42	30	72	112
,,	V.	640	Subcutaneously	6	3	9	89	3	92	101
Chloroacetylcysteine†	Pk.	240	Orally	17	0	17	75	5	80	97
Glycylcysteine	V.	288	Subcutaneously	43	4	47	9	3	12	59
,,	P.	320	,,	56	0	56	18	0	18	74
,,	P.	320	Orally	66	0	66	6	5	11	77
,,	V.	320	Subcutaneously	57	0	57	25	12	37	94
Reduced glutathione	V.	320	Orally	72	6	78	0	0	0	78
,,	V.	320	Subcutaneously	66	0	66	7	6	13	79
Oxidised glutathione‡	P.	320	,,	56	0	56	24	0	24	80
,,	P.	320	Orally	77	11	88	0	5	5	93

* With "low protein" diet. † Given in two doses.
‡ Diarrhoea, urine contaminated.

Discussion.

The work of Abderhalden and Samuely [1905] on the sulphur metabolism of dogs may be summarised as follows.

Substance	Amount given per day (g.)	Number of days	% in urine as sulphate	% in urine as neutral sulphur
Cystine (orally)	1·0	10	65	19
Dialanylcystine (O)	1·6	8	58	14
,, (subcutaneously)	1·6	5	38	24
Dileucylcystine (C)	1·9	2	33	29

Stearns and Lewis [1930] found that rabbits oxidised 92·8 % of a dose of cystine ethyl ester hydrochloride to sulphate in 2 days. We are aware of no other quantitative work on the metabolism of sulphur compounds of this type except the very thorough investigations by Lewis and co-workers and Sherwin and co-workers on derivatives of cystine containing an aromatic group. For our present purpose, on account of the aromatic group, that work is not strictly relevant.

It is clear from Table I that the extent to which a substance (except dichloroacetylcystine) is oxidised is very variable but is much the same whether it is given in the sulphydryl or disulphide form and whether it is given subcutaneously or orally. Our results may therefore be summarised as follows:

Substance	% excreted as sulphate in 2 days	% excreted as neutral sulphur in 2 days
Cystine and cysteine	69·7	3·7
Racemic cystine and cysteine	66·0	16·2
Acetylcysteine	42·7	40·7
Chloroacetylcysteine and dichloroacetylcystine	22·0	81·3
Glycylcysteine	56·5	19·5
Glutathione	72·0	10·5

The similarity between the figures for acetylcysteine, dialanylcystine, dileucylcystine and, to a lesser extent, glycylcysteine is interesting when compared with the relatively much greater extent to which cystine and its ester and glutathione are oxidised. It has now been shown definitely by Nicolet [1930] and by Kendall and co-workers [1930] that glutathione, as was thought, though on somewhat inadequate evidence [Pirie and Pinhey, 1929], in this laboratory, is glutamylcysteinylglycine. A study of the oxidation of the related peptides glutamylcysteine and cysteinylglycine would be interesting.

It is clear from the figures given that a dog can oxidise *r*-cystine to much the same extent as *l*-cystine; it appears that a greater proportion of the racemic compound is excreted as neutral sulphur. In no case has the nature of this neutral sulphur been investigated but we intend to do this at a later date. The percentage of the sulphur administered which is accounted for is greater in the case of inactive cystine or cysteine than in that of the normal

isomeride. This suggests that, as would be expected, the unnatural isomeride is not used to replace tissue breakdown.

The marked difference between the extent to which dichloroacetylcystine is oxidised when given orally and when given subcutaneously suggests that the substance is only oxidised after hydrolysis. In addition to oral administration chloroacetylcysteine was given subcutaneously after neutralisation. There was no rise in sulphate excretion but 84 % of the sulphur in the dose appeared as neutral sulphur. 2 mols. of sodium hydroxide are necessary to raise the p_H of a solution containing 1 mol. of chloroacetylcysteine to 7. The neutral solution contains chlorine ions, no amino-group is present and the sulphydryl group is unaffected. Presumably either hydroxyacetylcysteine or its anhydride is formed but this has not yet been demonstrated conclusively.

Summary.

The capacity of the dog to oxidise cystine and allied compounds, given orally or subcutaneously, has been studied.

The dog can oxidise to the same degree, *l*- and *r*-cystine and *l*- and *r*-cysteine and glutathione.

Acetylcysteine and glycylcysteine are not so readily oxidised.

Chloroacetylcysteine and dichloroacetylcystine are very resistant.

The nature of the neutral sulphur excreted as result of administering certain of the compounds has not yet been investigated.

The administration of glycylcysteine anhydride causes diuresis in dogs and rats but not in rabbits.

p-Iodophenylcysteine upsets the dog and is not well absorbed.

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[Note added 15. viii. 31.] As the neutral sulphur figure (24 %) in the experiment with injected oxidised glutathione appeared high, we repeated this experiment and certain others on the same dog, Patsy. Stocks being low, we used smaller doses, containing in each case 190 mg. sulphur. The results were as follows: oxidised glutathione given orally; excreted as sulphate 58 % and as neutral sulphur 6 %; given subcutaneously; 50 % and 12 %. Reduced glutathione given orally; 70 % and 0 %. *l*-Cystine given orally; 70 % and 0 %. The high figure in question, therefore, was certainly due to faecal contamination of the urine. The new figures support the argument in the discussion.

CXXVI. THE DETERMINATION OF VITAMIN A IN COD-LIVER OILS (*a*) BIOLOGICALLY, (*b*) CHEMICALLY, (*c*) PHYSICALLY, WITH A STATISTICAL EXAMINATION OF THE RESULTS.

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A method for the biological estimation of vitamin A has been described by Coward *et al.* [1930, 1931] which appears to have a greater degree of accuracy than the methods previously used. It therefore appeared desirable to use it for measuring the vitamin A values of several samples of cod-liver oil and to compare these values with the "blue values" obtained by the antimony trichloride (or arsenic trichloride) colour test originally put forward by Rosenheim and Drummond [1925] as a measure of the same factor. Eleven samples of oil and two concentrates were examined:

(*a*) biologically (Pharmaceutical Society);

(*b*) chemically by the colour test ($SbCl_3$) on (1) the oils and (2) their unsaponifiable fractions (Pharmaceutical Society—by F. J. Dyer);

(*c*) physically—the intensity of the 328 $m\mu$ absorption and the intensities of the 572 $m\mu$ and 606 $m\mu$ bands of the blue colour obtained with $SbCl_3$ on the oils (The University, Liverpool—by R. A. Morton).

The results were examined statistically by J. H. Gaddum of the National Institute for Medical Research.

The oils and concentrates which were examined by the three methods are given in Table I.

It is evident from Table VII in which the various results have been collected that, in general, an oil with a high biological value has also the following properties.

(*a*) A high blue value obtained by the action of $SbCl_3$ on the oil itself, though the two values are by no means parallel.

(*b*) A high blue value obtained by the action of $SbCl_3$ on the unsaponifiable fraction of the oil. These values are almost parallel.

(*c*) A high value for the absorption bands 572 $m\mu$ and 606 $m\mu$ obtained by the action of $SbCl_3$ on the oil. The intensities of these two bands run parallel in these oils, though Morton has examined numerous oils in which they do not.

Table I.

Designation	Source	Quality	Date of catch
A	Scotch	Finest non-freezing	1928
B	—	Cattle and poultry	1930
C	Norwegian	Finest non-freezing	1930
D	Icelandic*	Finest non-freezing	1930
E	Icelandic*	Finest non-freezing	1930
F	Icelandic*	Unrefined	1930
G	—	Unrefined (?)	1930 (?)
H	Norwegian	Finest non-freezing	1926
J	—	Cod, suspected of being adulterated with shark-liver oil	1928
K	Scotch and Norwegian (mixed)	Finest non-freezing	1930
L	—	Suspected of being adulterated (Unsap. M. = 1·9 %) (F.F.A. = 4·1 %) (I.V. = 152)	1930
M	Concentrate		
N	Concentrate		

* Received direct from the manufacturers, Messrs I. Spencer, Aberdeen.

(*d*) A high value for the intensity of the 328 $m\mu$ absorption band of the oil itself. These values are almost parallel.

There are, however, certain exceptions to this: *e.g.* oil C has a higher biological value than oil A (about 3 : 2) whereas its chemical and physical values are lower than those of oil A (average about 3 : 4); similarly oil K has a biological value about 2·5 times that of oil A and its chemical and physical values are practically equal to those of oil A. It was obviously necessary, therefore, to have the results examined as carefully as possible, partly to see what faith we could place on the biological tests and then to determine which chemical or physical measurement was generally the truest measure of the vitamin A content. Dr J. H. Gaddum has done this and having calculated the probable range of each test on the severe criterion of $\pm$ twice the standard deviation where $\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$, he found that many of the physical measurements lie outside the range of the known error of the biological test and suggested that certain oils might contain substances which were inactive biologically but which might increase the blue value ($SbCl_3$) and the intensity of the absorption band 328 $m\mu$. On the other hand, certain oils might contain physiologically active substances other than vitamin A, or the biological test itself might be subject to unknown errors. On the whole the intensity of the 328 $m\mu$ absorption band gave the best agreement with the biological value, and the blue value obtained by the action of antimony trichloride on the unsaponifiable fraction of the oil was nearly as good. Both of these measures were found to be much more accurate than the Lovibond measure of the blue value obtained with the oil itself.

BIOLOGICAL EXAMINATION OF THE OILS.

The vitamin A value of oil A has been very thoroughly examined and the result described in papers by Coward *et al.* [1930, 1931]. It was found that the relation between the dose of oil given and the mean increase in weight of a group of rats given the daily dose of oil for 5 weeks, after having become steady in weight on a diet deficient in vitamin A, was expressed by the equation:

$$y = 12{\cdot}1 + 40{\cdot}24 \log x,$$

where y equals the mean increase in weight in grams of the group in 5 weeks, and x equals the daily dose of cod-liver oil in mg. This relation held when the numbers of bucks and does in each group were approximately equal. The behaviour of the bucks and does in each group was also calculated separately, and the following equations obtained:

$$y = 11{\cdot}3 + 50{\cdot}3 \log x \text{ (bucks)},$$
$$y = 12{\cdot}4 + 27{\cdot}4 \log x \text{ (does)}.$$

The latter equations have now been used for making comparisons of the biological values of ten samples of cod-liver oil and two concentrates.

Rats were prepared for the test by giving them a diet consisting of:

Caseinogen, light-white (B.D.H.) non-extracted	15 %
Dextrinised rice starch	73 ,,
Dried yeast	8 ,,
Salt mixture (Steenbock's 40)	4 ,,

About 2 units of vitamin D were given to each rat twice a week. Each sample of cod-liver oil was so diluted with olive oil that the required daily dose was always contained in 1 drop of the solution. Daily doses were given by dropping directly into the rat's mouth from tubes selected to deliver not more than 21 mg. and not less than 19 mg. oil in 1 drop. Fresh dilutions were made every 7 days and kept in cold store when not actually in the rat room for feeding. Each sample of cod-liver oil was tested in 1–4 doses. Each dose was tested on large groups of animals (9–26), each animal of a group receiving the same daily dose for 5 weeks. The average increases in weight of the bucks and does of each group were then calculated separately, and, by substitution in the corresponding equation, the dose of oil A which would have brought about this increase in weight was determined. The inverse ratio of this dose to the actual dose of the oil under test is the ratio of the vitamin A contents of the oils. The potency of the fresh oil was then calculated as a percentage of that of oil A. The average of the results from bucks and does on all doses of an oil was taken as the measure of its vitamin A potency. In addition the percentage deviation of each group of bucks and does from the average was calculated. Results are collected in Table II. An analysis of the percentage deviations of the groups reveals a surprising degree of accuracy in the results. Twenty different doses of oils were tested, giving 40 results, as the bucks and does were calculated

Table II. *Biological examination of the oils. The vitamin A value of each oil and concentrate is expressed as a percentage of the vitamin A value of oil A.*

Deviation

Table II. *Biological examination of the oils. The vitamin A value of each oil and concentrate is expressed as a percentage of the vitamin A value of oil A.*

Cod-liver oil	Dose mg.	No. of bucks on test	Av. inc. in 5 weeks g.	Deviation from mean of each group $\sigma = \sqrt{\Sigma d^2/(n-1)}$ bucks	Dose of oil A which would bring about this inc. mg.	No. of does on test	Av. inc. in 5 weeks g.	Deviation from mean of each group $\sigma = \sqrt{\Sigma d^2/(n-1)}$ does	Dose of oil A which would bring about this inc. mg.	Vitamin A potency (%) relative to oil A according to bucks	Vitamin A potency (%) relative to oil A according to does	Weighted mean	Percentage deviation of each group from the average: bucks	Percentage deviation of each group from the average: does	Date at which each test was begun
B	1·0	10	4·2	10·2	0·723	9	11·8	11·3	0·95	72·3	95	—	− 7·8	+21·2	14. vii. 30
	4·0	4	35·75	12·7	3·06	5	24·6	7·2	2·79	76·5	69·7	—	− 2·4	−11·1	10. vi. 30
										Av. = 78·4		79·7			
C	0·5	7	−0·9	7·5	0·571	10	−0·8	11·6	0·33	114·2	66·0	—	−26·3	−57·4	10. x. 30
	1·0	6	27·7	11·3	2·118	13	20·2	5·1	1·9275	211·8	192·75	—	+36·7	+28·4	25. vii. 30
	5·0	13	61·4	18·3	9·91	4	37·25	11·8	8·075	198·2	161·5	—	+27·9	+ 4·3	10. iv. 30
	5·0	7	54·9	18·2	7·357	11	36·2	11·6	7·393	147·1	147·9	—	− 4·1	− 4·5	20. iii. 30
										Av. = 154·9		156·6			
D	0·5	8	2·5	7·6	0·6685	12	6·33	7·8	0·599	133·7	119·8	—	+ 3·0	− 7·7	21. x. 30
	1·0	8	15·0	13·8	1·185	11	17·0	14·5	1·472	118·5	147·2	—	− 8·7	+13·4	22. viii. 30
										Av. = 129·8		130·1			
E	0·5	10	19·2	12·0	1·436	16	9·4	15·2	0·777	287·2	155·5	—	+63·7	−11·4	23. x. 30
	1·0	10	18·0	11·13	1·358	11	14·9	8·5	1·233	135·8	123·3	—	−22·6	−29·7	17. ix. 30
										Av. = 175·45		171·8			
F	0·1	10	1·7	6·9	0·6442	11	9·8	16·4	0·8037	644·2	803·7	—	+ 2·7	+28·1	26. ix. 30
	0·5	8	40·4	18·4	3·787	12	27·2	8·9	3·4682	757·4	693·6	—	+20·7	+10·6	24. x. 30
	1·0	14	42·4	9·6	4·15	6	30·3	15·7	4·498	415·0	449·8	—	−33·8	−28·3	25. viii. 30
										Av. = 627·3		625·8			
G	1·0	8	18·4	12·2	1·384	8	18·5	4·4	1·671	138·4	167·1	—	− 9·4	+ 9·4	11. ix. 30
										Av. = 152·75		152·75			
H	1·0	10	9·7	12·1	0·929	9	7·0	11·1	0·635	92·9	63·5	—	+18·8	−18·8	25. ix. 30
										Av. = 78·2		78·9			
J	1·0	10	14·6	11·4	1·163	12	15·3	6·9	1·276	116·3	127·6	—	− 4·6	+ 4·6	19. ix. 20
										Av. = 121·95		122·5			
K	1·0	2	31·5	0·5	2·5212	8	22·8	8·72	2·3960	252·1	239·6	—	+ 2·5	− 2·5	4. xi. 30
										Av. = 245·8		247·1			
L	0·067	7	13·7	10·1	1·116	7	12·0	7·6	0·9670	1674·0	1450·5	—	+ 7·1	− 7·1	9. i. 31
										Av. = 1562·25		1562·25			
M	0·02	6	−4·0	17·4	0·49636	4	−2·0	6·96	0·29854	2482·0	1493·0	—	+24·9	−24·9	15. xii. 30
										Av. = 1987·5		2086·4			
N	0·04	5	1·8	6·1	0·6473	5	6·2	1·4	0·5939	1618·25	1484·75	—	+ 4·3	− 4·3	20. i. 31
										Av. = 1551·5		1551·5			

separately. Of these, two results only showed more than 50 % deviation (57·4 and 63·7 respectively) from the average value of the sample of oil; two results showed between 30 and 40 % deviation, eleven between 20 and 30, six between 10 and 20, and nineteen less than 10 %. It is, of course probable that, if more doses of each of the last seven oils or concentrates had been tested, the average would have been somewhat different and some of the percentage deviations correspondingly greater. The results on the whole support our claim that a group of about 10 rats used in a test in this manner will give a result almost always within ± 30 % of the true value. Hence, we claim that the biological values which we have assigned to these oils as the result of this method of testing on much larger numbers of rats must be very near to the true values for the oils.

CHEMICAL EXAMINATION OF THE COD-LIVER OILS.

By F. J. DYER.

The oils used in the biological investigation described above were subjected to the following chemical tests.

A. *The oils themselves.*

The chief values determined were:

(1) antimony trichloride blue value by comparison with Lovibond blue glasses;
(2) natural tint;
(3) acid value;

and the opportunity was taken for carrying out also the following tests:

(4) Fearon's reaction;
(5) phosphomolybdate test;
(6) trichloroacetic acid test.

For suggesting to me these three tests and for kindly advising me concerning the conditions under which he applies them in his own laboratory, I am greatly indebted to Dr O. Rosenheim, of the National Institute for Medical Research.

B. *Unsaponifiable fractions of the oils extracted by the method of Smith and Hazley [1930].*

Antimony trichloride blue value (Lovibond measure) and other tests applied to a chloroform solution of the unsaponifiable fractions.

Experimental.

A. *Examination of the oils.*

(1) *Antimony trichloride blue value (Lovibond).* This determination was made by the Carr and Price [1926] modification of the Rosenheim and Drummond test [1925], standardised to a greater degree of uniformity and precision

by the undermentioned conditions recommended by the Cod-liver Oil Colour Test sub-committee of the Pharmacopoeia Commission [1931]. It is well known that the relation between intensity of colour developed and concentration of an oil is not linear but is represented by a line which falls away at higher concentrations to a curvilinear function. For the final colour values of an oil the concentration was chosen so that readings were made between the Lovibond blue figures 4 and 6, a range over which for most oils intensity of colour produced bears an approximately linear relationship to the concentration. Blue values were, however, taken for other concentrations in order that curves for each oil might be constructed if desired.

The conditions under which the test was carried out were as follows.

Reagent. A solution of antimony trichloride was prepared in pure alcohol-free chloroform, saturated at 20°, and found to contain 23 % $SbCl_3$ immediately before use. It was stored in a stoppered amber-coloured glass bottle.

Procedure. Two grams of oil were weighed into a narrow-necked 10 cc. measuring flask, and the flask filled to the mark with chloroform at 20° and mixed.

0·2 cc. of the solution was delivered from a 1 cc. burette, the graduated portion of which measured 16 cm., into a rectangular cell of 1 cm. in the direction of observation as described below. 2 cc. of antimony trichloride reagent were added, the solutions allowed to mix, and the maximum blue colour was immediately matched in a tintometer by Lovibond glasses. Several readings were taken on each oil to ensure that the maximum intensity had been recorded.

Samples of six of the oils were also distributed to three other laboratories and similar determinations to the above were made under the same conditions.

(2) *Natural tint.* The colour of the oils was considered of importance in view of the statement of Drummond and Hilditch [1930] that "a yellow oil is probably richer in vitamins than one paler in colour" if freshly prepared, and even when old, provided that proper precautions have been taken in the making to remove all traces of liver tissue and accompanying enzymes.

The natural tint was determined by placing some oil in a tintometer cell measuring 1 cm. in the direction of observation and matching the colour as seen by diffused daylight with Lovibond yellow and red glasses in a tintometer.

(3) *Acid value.* The importance of a medicinal cod-liver oil possessing a low acid value, preferably below 0·3 % of free fatty acids calculated as oleic acid, has been emphasised by Drummond and Hilditch [1930]. It is also of importance in helping to determine how far the yellow tint of the oil may be regarded as significant, for a high acid value would indicate the presence of fat-splitting enzymes in accompanying liver tissue which would darken the oil.

Free acidity was determined in the customary manner by titration of a weighed quantity of oil in hot neutral alcohol with $N/10$ potassium hydroxide

using phenolphthalein (1 % in alcohol) as indicator. The percentage of free fatty acid was calculated as oleic acid.

(4) *Phosphomolybdate test.* When a solution of cod-liver oil in chloroform or light petroleum is shaken with a solution of sodium phosphomolybdate (10 %) in nitric acid (5 parts strong acid with 95 parts water), and, after allowing the mixture to stand, a strong solution of ammonia (sp. gr. 0·880) is added to the upper layer, a blue colour is developed. As the intensity of the colour is influenced by the time of shaking and by the length of the interval before adding the ammonia, the arbitrarily-chosen conditions set out below were adopted for each oil examined.

Method. To 1 cc. of 20 % solution of cod-liver oil in chloroform (or such other concentration as to give readings between 4–6 blue units as before), contained in a 10 cc. stoppered cylinder were added 2 cc. reagent. The mixture was shaken for exactly 10 seconds, allowed to stand for 1 minute, and then 3 cc. strong ammonia added and allowed to mix with the upper green layer. The maximum blue colour produced was read in a Lovibond tintometer in a cell measuring 1 cm. in the direction of observation using diffused daylight.

There is no evidence that the colour produced in this reaction bears any relation to vitamin activity, but it was thought to be worth while to determine whether there existed any relation between this chromogenic substance and the intensity of blue colour produced by the same oil with antimony trichloride.

(5) *Trichloroacetic acid test.* When trichloroacetic acid is shaken with a chloroform solution of cod-liver oil or preferably with a solution of its unsaponifiable matter, a blue colour is produced, and it was thought to be of interest to compare the intensities of the colours given under standard conditions by the cod-liver oils under investigation. The reagent for the test was prepared by mixing together:

Trichloroacetic acid (A.R.)	100 g.
Distilled water	10 cc.

allowing to stand overnight and using the resulting clear solution. The blue colour produced was measured in exactly the same manner as that described under the antimony trichloride test, using 2 cc. reagent and 0·2 cc. of a solution of the oil in chloroform of such strength that the range of Lovibond blue colour was between 4 and 6. It was thought that this colour, which is usually intensified after saponification of the oil, might bear some relationship to the antimony trichloride blue colour.

(6) *Fearon's Reaction.* Fearon [1925] using the converse of a test devised by Whitby [1923] for detecting sterols, claimed that the rose-pink colour produced in the presence of a condensing agent by the addition of a polyphenol, such as pyrogallol or resorcinol, to a chloroform solution of cod-liver oil was a measure of vitamin A potency. This claim was refuted by Rosenheim and Webster [1926], who showed that unsaponifiable matter of cod-liver oils in which vitamin A was abundantly present failed to give Fearon's reaction.

Further, they regenerated the chromogenic substance from the lead soaps of the unsaturated fatty acids of the original oil, and from other evidence suggested that the chromogen was probably an aldehydic oxidation product of an unsaturated acid (*e.g.* of the clupanodonic series). It was, therefore, considered of interest to apply the test to the oils under examination to see whether any relationship existed between this chromogen and the unsaturated fatty acids to which Norris and Church [1930] and others attribute interference with the blue colour produced by antimony trichloride with cod-liver oils at higher concentrations.

The method used was as follows.

To 0·5 cc. of 20 % solution of cod-liver oil in chloroform contained in a 10 cc. graduated stoppered cylinder was added sufficient trichloroacetic acid solution (12 % in light petroleum) to make 5 cc. A few crystals of pyrogallol were added and the mixture well shaken at intervals during 30 minutes. Another similar cylinder was prepared, containing in addition to the above mixture 3 drops (delivered from a standard dropper and each weighing 0·02 g.) of 1 % solution of purified benzoyl peroxide. (Commercial benzoyl peroxide was dissolved in chloroform and precipitated by methyl alcohol.) The rose pink colour produced was measured, using diffused daylight in a Lovibond tintometer, the mixture being contained in a cell measuring 1 cm. in the direction of observation. Any difference between the tints with and without added peroxide may give some indication of the peroxide content of the oil. The reagent was prepared by diluting with three parts of light petroleum a saturated solution of trichloroacetic acid (A.R.) in light petroleum, the solvent being previously dried over concentrated sulphuric acid as suggested by Fearon.

Results.

The values obtained by the above investigation are summarised in Table III. It may be seen that none of the determinations by the phosphomolybdate test, the trichloroacetic acid test or Fearon's reaction can be considered as a measure of the substance measured by the antimony chloride test.

In Table IV are the results obtained by four different laboratories for six of the oils examined by the antimony trichloride test under rigid conditions to ensure as close uniformity as possible. Although their individual results differ widely, the order of potency of the oils (Table IV), obtained by each of the four workers, is almost the same except for oils B and H. At another time by special request, one of these workers (N. E.) examined oil F and recorded a blue value between 70 and 90, which is in close agreement with the writer's finding of 86.

B. *Preparation and examination of the unsaponifiable fractions of these oils.*

The unsaponifiable fractions of the oils were prepared by the rapid method described by Smith and Hazley [1930], in which 2 g. of the oil are saponified

by 10*N* potassium hydroxide in an open tube, and the unsaponifiable residue is extracted by repeated shakings with chloroform until the chloroform washings from the soap solution no longer give a blue colour with arsenic trichloride.

The supply of oils D, E and F was not sufficient to admit of their unsaponifiable residues being prepared.

The technique was slightly modified by the addition of a few drops of alcohol to the second and third extractions to effect more rapid separation of the mixture into two layers.

(1) *Antimony trichloride blue value.* The residues obtained by evaporation of the mixed chloroform extracts in the above method were dissolved in chloroform to a volume 5, 10 or more cc. according to the known blue value of the oil under examination. The proportion of unsaponifiable matter was not determined, but the results given in Table V are expressed as values of unsaponifiable matter equivalent to the 2 g. of original oil used in its preparation. As a rule, the results show that when readings are taken at the 4 to 6 range of Lovibond glasses and converted to the 20 % concentration, the unsaponifiable matter gives a higher blue value than the amount of oil from which it was prepared. This is in accord with the results of Smith and Hazley.

It may be further observed that whereas for oils of lower antimony trichloride values the relationships to oil A of their blue values after saponification have not greatly altered from the same relationships before saponification, this does not hold true for oils such as F and L of higher blue values. Apparently the somewhat drastic method of saponification has caused considerably more loss of chromogen in these oils than in other oils.

(2) *Fearon's reaction.* Concentrates of some oils unexpectedly gave traces of the Fearon reaction, which appears to show that by this method, saponification is not always complete. No consistent parallelism between Fearon-values

Table III. *Constants and colour values of cod-liver oils used in the biological investigation.*

Oil	Natural tint		Acid value % F.F.A.	Antimony trichloride test. Value calculated for 20 % concentration of oil. Lovibond blue	Phosphomolybdate test. Value calculated for 20 % concentration of oil. Lovibond blue	Trichloroacetic acid test. Value calculated for 20 % concentration of oil. Lovibond blue	Fearon's reaction	
	Lovibond yellow	Lovibond red					Without benzoyl peroxide	With benzoyl peroxide
A	1·8	0	0·34	9·0	1·5	2·7	0·5	0·7
B	3·5	0·3	2·27	4·4	4·7	Nil	12·0	12·5
C	1·2	0·2	0·17	7·6	Trace	1·0	0·4	5·5
D	2·5	0·2	0·38	10·8	—	—	—	—
E	1·6	0·1	0·2	15·2	—	—	—	—
F	8·9	0·8	2·89	86·0	—	—	—	—
G	2·4	0·3	0·38	28·0	Trace	3·9	6·7	11·2
H	1·3	0·1	0·24	4·3	2·3	Trace	2·1	2·3
J	9·2	1·2	1·54	17·4	Trace	2·8	11·7	21·1
K	1·2	0	0·28	10·2	3·5	1·5	2·2	4·0
L	12·0	1·4	4·0	305	Nil	104	Nil	Nil

and blue values obtained by other tests, has been detected during this investigation.

(3) *Trichloroacetic acid test.* One feature of incidental interest is that the blue value given by trichloroacetic acid is greater for a concentrate than for the amount of oil from which it was prepared (Tables III and V). It is possible that an interfering substance has been removed during saponification and that thereby the chromogen is enabled to exert its full power. For concentrates A, B, C, G and H it will be seen that the blue values given by the trichloroacetic acid test run roughly parallel with values given by those concentrates with antimony trichloride.

Table IV. *Antimony trichloride Lovibond blue values of six of the cod-liver oils obtained by four different workers. (Calculated for 20 % dilutions of the oil.)*

Oil	H. A. J.	N. E.	T. T. C.	F. J. D.
A	11·2	11·1	12·4	9·0
B	4·4	4·4	4·7	4·4
C	8·1	8·0	10·0	7·6
G	24·0	24·0	30·6	28·0
H	5·7	5·1	4·1	4·3
J	16·0	13·8	24·0	17·4

It will be seen that although there is a wide variation in these figures, there is a fairly good agreement as to the ascending order of potency, which may be expressed thus:

Order of potency (Lovibond blue) of the six oils according to:

H. A. J.	B H C A J G
N. E.	B H C A J G
T. T. C.	H B C A J G
F. J. D.	H B C A J G

Table V. *Colour values given by unsaponifiable residues of cod-liver oils of Table III, and by two concentrates.*

Note. The unsaponifiable residues were prepared by the method of Smith and Hazley using 2 g. of oil.

Oil	Antimony trichloride test. Value calculated for 20 % concentration of original oil. Lovibond blue	Phosphomolybdate test. Value calculated for 20 % concentration of original oil. Lovibond blue	Trichloroacetic acid test. Value calculated for 20 % concentration of original oil. Lovibond blue	Fearon's test (with added benzoyl peroxide). *Note.* For unsaponifiable residues this should be negative (see text). Lovibond red
A	17·25	1·7	8·5	1·6
B	8·0	8·3	4·2	1·2
C	12·9	1·2	5·3	1·0
D	21·2	—	—	—
E	27·0	—	—	—
F	100·0	—	—	—
G	42·0	2·0	24·6	1·2
H	12·6	1·4	7·2	1·0
J	26·6	1·0	8·8	Nil
K	19·6	2·1	17·2	Trace
L	266·0	Nil	230·0	Nil
M	420·0	—	—	—
N	290·0	—	—	—

PHYSICAL EXAMINATION OF THE OILS.

By R. A. MORTON.

All the materials tested biologically have been examined as carefully as possible by the available physical methods. The spectroscopic measurements on the blue colours were carried out on the reagent as described by Carr and Price. The investigation of ultra-violet absorption followed the technique described by Morton and Heilbron [1928]. No corrections have been introduced. The notation $E^{1\%}_{1\,cm.}$ refers to the intensity of absorption given by a 1 % solution of the material to be tested using a 1 cm. cell, the concentration referring to the actual solution studied in the spectroscope. E is defined by $\log I_0/I = E$, I_0 being the intensity of the incident light and I that of the emergent light (entering and leaving the absorption cell).

It must be emphasised that, since the absorption at 328 $m\mu$ is not wholly due to one substance, the values for $E^{1\%}_{1\,cm.}$ are all likely to be high compared with the intrinsic absorption of vitamin A (if it proves to be such). The correction to be applied is, however, very small in some fresh cod-liver oils but must differ from oil to oil. In concentrates the magnitude of the correction remains equally uncertain. Instead, therefore, of introducing any corrections we have used the gross intensity of absorption at 328 $m\mu$. The instrumental error is about $\pm$ 3 % in this work, but the uncertainty as to the magnitude of the correction may raise the gross experimental error in extreme cases to $\pm$ 10 %.

With regard to the spectroscopic measurement of the absorption at 572 and 606 $m\mu$ in the blue solutions, it must be emphasised that none of the oils was very fresh when received for spectroscopic examination. The 606 $m\mu$ chromogen (arising in part from the spontaneous oxidation of a substance occurring in many oils [Heilbron, Gillam and Morton, 1931]) was always present in reasonable quantity, so that the 572 $m\mu$ absorption could not be seen visually in the spectroscope as a definite band. The readings were made by setting the spectroscope to 572 $m\mu$ and determining the intensity of absorption irrespective of whether a clear absorption band could be seen or not. There is almost certainly some degree of overlapping between the two absorption bands with their maxima at 572 $m\mu$ and 606 $m\mu$ respectively. The values for $E^{1\%}_{1\,cm.}$ 572 $m\mu$, therefore, may require some correction, but as we have no exact basis for this the values have not been adjusted. The gross value for $E^{1\%}_{1\,cm.}$ 572 $m\mu$, does not, however, appear to be very inaccurate; the experimental error may be $\pm$ 10 %.

From other investigations [Gillam and Morton, 1931; Heilbron, Morton and Gillam, 1931] we are quite satisfied that, in extreme cases, the agreement between the 606 $m\mu$ band and either the 572 $m\mu$ or 328 $m\mu$ bands may be very much worse than is indicated by the data published in this paper.

Results.

The results of the physical examination of the oils are summarised in Table VI.

Table VI.

Oil	Date of examination	$E^{1\%}_{1\,cm.}$ 328 $m\mu$ ultra-violet	$E^{1\%}_{1\,cm.}$ 572 $m\mu$ spectroscopic	$E^{1\%}_{1\,cm.}$ 606 $m\mu$ colour test
A	December, 1930	0·78	0·70	0·92
A	April, 1931	0·80	0·75	1·04
B	December, 1930	0·60	0·37	0·47
C	December, 1930	0·67	0·50	0·61
D	December, 1930	0·935	0·92	1·14
D	April, 1931	0·90	0·74	0·95
E	December, 1930	1·03	1·02	1·35
E	April, 1931	1·0	0·80	1·09
F	December, 1930	4·3	3·81	4·76
G	December, 1930	1·68	1·45	1·92
H	December, 1930	0·46	0·33	0·33
J	December, 1930	1·30	1·28	1·50
K	December, 1930	0·85	0·68	0·85
L	April, 1931	8·0	7·5	12·15
M	April, 1931	13·0	15·3 (583 $m\mu$)	21·2 (620 $m\mu$)
N	April, 1931	10·0	9·14 (583 $m\mu$)	11·4 (620 $m\mu$)
Estimated experimental error (gross)		±10 %	±10 %	±10 %

It is clear that a very close agreement occurs between the 328 $m\mu$ absorption and the 572 $m\mu$ absorption. A similar agreement occurs in these samples between the 328 $m\mu$ and the 606 $m\mu$ absorption bands, though the small deviations which exist between the 328 and 606 $m\mu$ bands on the one hand and between the 328 and 572 $m\mu$ bands on the other lie in opposite directions. This agreement between the 328 and 606 $m\mu$ bands is probably misleading, for it has been shown [Gillam and Morton, 1931] that, in many samples of cod-liver oil, there is close agreement between the 328 and 572 $m\mu$ bands but wide disagreement between the 328 and 606 $m\mu$ bands.

STATISTICAL EXAMINATION OF RESULTS.

By J. H. GADDUM.

The results obtained have been examined statistically in order to extract information as to the relative accuracy of the different methods of measuring the concentration of vitamin A, and in order to discover whether the discrepancies shown by some of the oils could be attributed to the known error of sampling of the biological assay.

When the error to be expected in the measurement of any given quantity is proportional to the quantity itself, this error is usually expressed as a percentage of the result. The percentage standard deviation, which is sometimes called the coefficient of variation, is a convenient index of accuracy so long as it is small compared with 100, but when it is large its exact interpretation becomes ambiguous. An error of + 100 % or more may occur in a biological assay, but the expression "an error of − 100 %" has no useful meaning. When

the error is not small compared with 100 % it is clearly not desirable to express it as a percentage at all. Another difficulty arises from the doubt as to whether the error should be calculated as a percentage of the observed value or of the true value, and from the difficulty of carrying out the calculation in the latter case.

These difficulties disappear when the distribution of the logarithms of the readings is studied instead of that of the readings themselves. The error of the test can then be directly expressed as the error of the logarithm of the reading. In the present case the standard deviation of the logarithms (to base 10) of the results has been calculated and the square of this quantity is referred to as the "logarithmic variance." The meaning of the result expressed in this way is not immediately intelligible to those who are not accustomed to this form of expression, but when the calculations are otherwise complete, the result can easily be put in a more readily intelligible form. This method of calculation has been adopted here because, in cases like this, it is simpler, less ambiguous, and probably more rational than the calculation of coefficients of variation. For this purpose it is assumed that the logarithms of the readings are normally distributed. Arguments were put forward by Galton [1879] in favour of the view that, especially in the case of vital phenomena, this assumption was more rational than the usual assumption that the readings themselves were normally distributed. When the error is small compared with the actual readings, the two assumptions give almost identical results, and the chief reason that the former assumption is not generally adopted is probably that, in many cases, it would increase the complexity of the calculations without appreciably affecting the final result [Yule, 1924, Chap. VII, Par. 26].

The sampling error of the biological test.

The sampling error is due to the fact that, even when the conditions are so carefully controlled that the average response of the rats to the vitamin is constant, different individual rats will give different responses. The error is diminished by taking the average response of a number of rats, and its size is dependent on the number of rats used.

The standard deviation (σ) of the response of individual rats (increase of weight) has already been worked out from the results obtained with oil A and published [Coward *et al.* 1930]. These results appear to justify the assumption that this standard deviation is independent of the dose of cod-liver oil used. In order to obtain the best possible estimate of σ the published figures have been recalculated, using the equation $\sigma^2 = \frac{\Sigma d^2}{n-1}$ where $d =$ the deviation of each individual response from the mean response, and $n =$ the number of animals. This formula is likely to give a more accurate estimate than that previously used. The most probable value of σ^2 was found by taking the weighted mean of the different values of σ^2 thus obtained. The weight of each individual estimate of σ^2 was taken to be proportional to n. Separate estimates of the value of σ

for male rats and for female rats have also been obtained by applying the same method to the results with oils B–N. The estimates obtained by these calculations are shown in Table VIII.

It has been found that the response bears a linear relationship to the logarithm of the daily dose. If therefore the distribution of the response is normal, the distribution of the logarithm of the result of the assay will also be normal. A series of biological assays carried out in this way thus probably provides a good example of a series of readings of which the logarithms are normally distributed. This is the kind of distribution implied by the assumptions made in this paper and discussed above.

The standard deviation of the logarithm of the result (λ_1) is obtained by dividing σ by the slope of the line connecting the response with the logarithm (to base 10) of the dose. This is shown in the last column in Table VIII. Since the slope of the curve for male rats is greater than that for female rats, the error of the result is smaller when male rats are used than when female rats are used. It is therefore preferable to use male rats for this test when possible. The largest source of error in these estimates of λ_1 probably lies in our estimate of the slope of the curve connecting dose and effect. The data were considered insufficient for a satisfactory estimate of this error. The error of each test has therefore been calculated on the assumption that $\lambda_1 = 0{\cdot}4$ for all the rats used, and has been taken to be equal to $\frac{0{\cdot}4}{\sqrt{n}}$. It is reasonable to assume that the estimate obtained in this way is larger than the true sampling error of the test. Nevertheless it is found that the errors so calculated are not large enough to account for the discrepancies which occur between physical and biological estimates.

The limits between which the biological result may be expected to vary owing to the error of sampling are shown in Table IX. These limits correspond to twice the standard error; the two values given for each oil were obtained by first dividing and then multiplying the biological estimate by the antilogarithm of $\frac{2\times 0{\cdot}4}{\sqrt{n}}$, where n = the number of rats used. The potency corresponding to the standard curve has been taken as 100. This is probably equal to the potency of the oil A. The error in the estimate of the potency of the oil A from the curve has been calculated in the same way as the errors for the other oils.

The relative accuracy of the different methods of estimating vitamin A.

The results on which these calculations are based are shown in Table VII. The ordinary methods of calculating correlation or regression coefficients are not suitable in this case, because they give undue weight to the results obtained with the more potent oils. The method which has been used depends on calculating the distribution of the logarithms of the results. Some general arguments justifying the assumptions involved have been given.

Let x be any one of the figures in column I of Table VII, and y the corresponding figure in column II. If the two tests both give results directly pro-

Table VII. *Biological, chemical and physical measurements of 11 samples of cod-liver oil and 2 concentrates.*

Oil	Biol. value relative to oil A (=100) I	Blue value, $SbCl_3$ test. Lovibond measure: Oil II	Blue value, $SbCl_3$ test. Lovibond measure: unsap. fraction III	Blue value, $SbCl_3$ test. Spectrophotometric measure: 572 mμ IV	Blue value, $SbCl_3$ test. Spectrophotometric measure: 606 mμ V	Ultraviolet band 328 mμ oil itself VI
A	100	9·0	17·25	0·70	0·92	0·78
B	78·4	4·4	8·0	0·37	0·47	0·60
C	154·9	7·6	12·9	0·50	0·61	0·67
D	129·8	10·8	21·2	0·92	1·14	0·935
E	175·5	15·2	27·0	1·02	1·35	1·03
F	627·3	86·0	100·0	3·81	4·76	4·3
G	152·7	28·0	42·0	1·45	1·92	1·68
H	78·2	4·3	12·6	0·33	0·33	0·46
J	122·0	17·4	26·6	1·28	1·50	1·30
K	245·8	10·2	19·6	0·68	0·85	0·85
L	1562·3	305·0	266·0	7·5	12·15	8·0
M (Conc.)	1987·5	—	420·0	15·3 (583 mμ)	21·2 (620 mμ)	13·0
M (Conc.)	1551·5	—	290·0	9·14 (583 mμ)	11·4 (620 mμ)	10·0

portional to the vitamin A content of the oil, x/y should be constant for the different oils. In order to test how far this is so $(\log_{10}x - \log_{10}y)$ was calculated for each oil. The values obtained, which would be all equal if the error of both tests was zero, were found to vary considerably. The mean and standard deviation of $(\log_{10}x - \log_{10}y)$ were calculated.

Table VIII. *Showing estimates of the standard deviation* (σ) *of the response (increase of weight), and the standard deviation* (λ_1) *of the logarithm of an estimate of the daily dose based on an experiment with one rat.*

	Total number of rats used for the estimate	σ	λ_1
Male and female rats used in experiments with oil A	156	13·2	—
Male rats used in experiments with oils B–N	163	13·4	0·268
Female rats used in experiments with oils B–N	184	10·6	0·386

The mean provides an estimate of the factor by which the blue value of crude oil should be multiplied in order to find the biological activity. This factor has been calculated for all the chemical and physical tests and the results are given in Table IX. In this Table all the different determinations have been reduced to the units in which the biological results are stated by multiplying each figure by the appropriate factor determined in this way. It will be seen that many of the physical measurements lie outside the range of the known error of the biological test. In particular the physical methods appear to have over-estimated the potency of oils G and J and under-estimated those of oils C and K.

If $\lambda =$ the standard deviation of $(\log_{10}x - \log_{10}y)$, λ^2 gives an estimate of the combined logarithmic variances of the physical and biological methods of estimating vitamin A. If the logarithmic variance of sampling (λ_1^2) represents

Table IX. *Showing the results of the different tests all reduced to the same units and the probable limits of error of the biological test (probability = 21/22). Oils arranged in order of biological activity.*

	Biological value I		Blue value $SbCl_3$ test. Lovibond measure		Blue value $SbCl_3$ test. Spectrophotometric		
Oil	Most probable	Possible range ($\pm 2\lambda$)	Crude oil	Unsap. fraction	572 $m\mu$	606 $m\mu$	Ultra-violet band 328 $m\mu$ oil itself
	I		II	III	IV	V	VI
H	78	51– 119	48	81	57	46	70
B	78	55– 111	49	52	64	65	92
A	100	86– 116	101	111	121	127	120
J	122	82– 181	195	171	221	207	199
D	130	97– 174	121	137	159	157	143
G	153	96– 242	314	271	250	265	257
C	155	125– 193	85	83	86	84	103
E	175	134– 230	170	174	176	186	158
K	246	137– 440	114	126	117	117	130
F	627	495– 794	964	644	656	657	659
N	1551	866–2778	—	1870	1575	1572	1533
L	1562	954–2557	3418	1714	1293	1676	1227
M	1987	1110–3560	—	2170	2637	2924	1993
Factors by which readings in Table VII were multiplied to obtain the above results			11·2	6·442	172·3	137·9	153·3

the whole error of the biological test, the logarithmic variance of the physical test will be equal to $(\lambda^2 - \lambda_1^2)$. This has been calculated for each of the physical tests and the results are shown in Table X. The value taken for λ_1^2 was the

Table X. *Showing estimates of the logarithmic variance of the different tests.*

			Blue value $SbCl_3$ test				
			Lovibond measure		Spectrophotometric measure		
		Biological value	Crude oil	Unsap. fraction	572 $m\mu$	606 $m\mu$	Ultra-violet absorption at 328 $m\mu$ of oil itself
		I	II	III	IV	V	VI
Biological test	λ_1^2	0·0080	—	—	—	—	—
Ratio of biological to physical results	λ^2	—	0·0554	0·0252	0·0340	0·0328	0·0194
Physical results	$\lambda^2 - \lambda_1^2$	—	0·0474	0·0172	0·0260	0·0248	0·0114
Percentage range corresponding to $\pm 2\sqrt{\lambda^2 - \lambda_1^2}$ (within which the result may be expected to lie 21 times out of 22)		66–151	37–273	55–183	48–210	48–206	61–163

mean of the values for the tests on the different oils. These results have been put in a more familiar form by calculating the range (corresponding to twice the standard error) within which the result may be expected to lie 21 times out of 22, expressed as a percentage of the true value.

The fact that the apparent errors of the physical tests, measured in this

way, are much larger than the errors of the physical measurements themselves might be explained in more than one way. Some oils might contain substances which were biologically inactive and yet gave a blue colour with $SbCl_3$ and absorption in the ultra-violet, while other oils might contain substances which masked the vitamin A in both these physical tests. Both kinds of physical test might measure the concentration of some substance other than vitamin A which has a roughly similar distribution. It is also possible that the biological test is subject to unknown errors due, for example, to a variation in the sensitivity of the whole stock of rats, or to the presence in some oils of physiologically active substances other than vitamin A. It is hoped to test this latter possibility by means of further experiments.

The estimates of the logarithmic variance in Table X place the tests in order of their accuracy when applied to the particular oils used in these experiments, but these estimates are themselves subject to an error of sampling dependent on the number of oils tested. The significance of the difference between these estimates of the accuracy of the different tests has been studied by the methods given by Fisher [1930, Par. 41]. By comparing the estimates of λ^2 it has been found that the probability that the test in column VI is more accurate than that in column II is slightly greater than 0·95. Similar calculations, also based on the estimates of λ^2, show that the probability that the test in column III is more accurate than that in column II is about 0·8.

When allowance is made for the sampling error of the biological test by comparing the values of $(\lambda^2 - \lambda_1^2)$ both these probabilities are greatly increased. The value of λ_1^2 is not known with sufficient accuracy to warrant a precise estimate of the probability, but there is clearly a quite definite presumption in favour of the conclusion that the measurement of the ultra-violet absorption of the oil itself and the measurement of the Lovibond blue value for the unsaponifiable fraction both provide more accurate estimates of vitamin A than the Lovibond blue value for the crude oil.

Discussion.

For practical purposes the possibility of accepting any one of the chemical or physical measurements of the oil as a measure of its vitamin A potency may be determined from an examination of Table XI.

It is evident that the six oils and two concentrates collected in the upper part of Table XI show good agreement between the biological value, the blue value of the unsaponifiable fraction and the intensity of absorption at 328 $m\mu$. Three only of these oils show agreement between biological value and blue value of the oil itself. The five oils collected in the lower part of Table XI cannot be said to show any degree of agreement between biological and physical measurements that would serve any useful purpose, though one of these oils (B) shows better agreement between biological value and the intensity of its absorption at 328 $m\mu$ than between its biological value and any of its other

Table XI.

Oil	Biological value	Blue value of oil	Blue value of unsap. fraction	Intensity of absorption at 328 $m\mu$
H	78	48	81	70
A	100	101	111	120
D	130	121	137	143
E	175	170	174	158
F	627	964	644	659
N	1551	—	1870	1533
L	1562	3418	1714	1227
M	1987	—	2170	1993
B	78	49	52	92
J	122	195	171	199
G	153	314	271	257
C	155	85	83	103
K	246	114	126	130

physical values. It would, therefore, appear to be quite unsafe to accept the blue value of the oil itself as a measure of its vitamin A value. Both the blue value of the unsaponifiable fraction of an oil and the intensity of absorption at 328 $m\mu$ appear to be more often in agreement with the biological value, but these also may be widely divergent, a fact which would appear to indicate that certain oils may contain substances which increase the blue value and other oils may contain substances which decrease the blue value.

The statistical examination of the results has strengthened this opinion.

It may be significant that the oils for which we have found the best agreement between biological and physical measurements are those which we have received direct from manufacturers and which are known to be pure cod-liver oils of recent preparation.

Summary.

Eleven samples of cod-liver oil and two concentrates have been examined and the following values determined.

1. Biological value; vitamin A content as measured by resumption of growth in rats whose reserves of this factor had been exhausted by feeding on a diet deficient in vitamin A.

2. Blue value; obtained by the action of antimony trichloride on the oil itself and measured by the Lovibond tintometer.

3. Blue value; obtained by the action of antimony trichloride on the unsaponifiable fraction of the oil and measured by the Lovibond tintometer.

4. Intensity of absorption at 572 $m\mu$, using the blue solution obtained by the action of antimony trichloride on the oil itself, measured spectrophotometrically.

5. Intensity of absorption at 606 $m\mu$, using the blue solution obtained by the action of antimony trichloride on the oil itself, measured spectrophotometrically.

6. Intensity of absorption at 328 $m\mu$ for the oil itself, measured spectrophotometrically.

Statistical examination of the results shows:

(*a*) that the discrepancies between the physical and biological measurements are much larger than the known sampling error of the biological test;

(*b*) that, of the physical and chemical measurements, the best agreement with the figures determined by the biological method was given by the measurement of the 328 $m\mu$ absorption band, and nearly as good agreement was given by the Lovibond blue value of the unsaponifiable fraction of the oil;

(*c*) that both the 572 $m\mu$ and 606 $m\mu$ bands gave less good agreement;

(*d*) that the Lovibond blue values obtained from the oils themselves give the least good agreement and it would appear to be unwise to rely upon this method for the measure of vitamin A in an oil. It can only be used as a very crude indication of vitamin A potency;

The oils whose biological values most closely agree with the values of the 328 $m\mu$ band and the Lovibond blue values of the unsaponifiable fractions are those which were received direct from the manufacturers and examined within a few months of their preparation.

We have to thank Messrs Isaac Spencer and Messrs Allen and Hanbury for supplying samples of oil; Miss B. G. E. Morgan for help given in the biological part of this work and Mr H. W. Ling for help given in the chemical part.

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CXXVII. TRYPANOCIDAL ACTION AND TOXICITY TO ENZYMES.

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It has been shown in a previous communication [Quastel, 1931] that the Congo red series of dyestuffs is highly toxic to the enzyme fumarase, prepared either from bacteria or from mammalian tissues. The presence of proteins greatly affects the toxicity of the dyes; this is due to the adsorption or combination of the dyestuff with protein, there resulting a diminution in the concentration of free dyestuff. With fumarase extracted from brain tissue and prepared in such a way as to be relatively free from protein and phosphate, Congo red is toxic at a molar concentration of $1{\cdot}2 \times 10^{-8}$.

Trypan-blue is highly toxic and it has now been found that trypan-red is also toxic, though its activity is not as great as that of the former. It is not to be supposed that these substances are general enzymic poisons, for experiment has shown that neither Congo red nor trypan-blue has any toxic action upon the enzyme urease prepared from Soya bean. It is evident that there must be some close connection between the structure of fumarase and that of the Congo red series of dyestuffs, which allows this specificity of behaviour to obtain.

The fact that trypan-blue and trypan-red have highly toxic actions upon fumarase led the writer to consider the possibility that other trypanocidal agents, not belonging to the Congo red series, might affect the activity of fumarase. Accordingly, Bayer 205 was tested and it has been found that this substance is also very toxic, its behaviour, as in the case of Congo red and trypan-blue, being specific, for it has no toxic effect on urease.

It is necessary now, for a proper appreciation of what follows, to give a brief résumé of recent work on the mechanism of trypanocidal action.

Following the discovery by Ehrlich and Shiga that trypan-red (I),

(I) $C_{10}H_4(SO_3H)_2(NH_2).N:N.C_6H_4.C_6H_3(SO_3H).N:N.C_{10}H_4(NH_2)(SO_3H)_2$,

a member of the Congo red series of cotton dyes, can cure an experimental infection of trypanosomiasis in mice, Nicolle and Mesnil examined a large number of cotton dyes belonging to the Congo red series and found that the substance having the best curative action on infection with *Trypanosoma gambiense* was a *s*-carbamide (II) derived from H-acid.

(II) $C_{10}H_3(SO_3H)_2(NH_2)(OH).N:N.C_6H_4.NH.CO.NH.C_6H_4N:N.C_{10}H_3(SO_3H)_2(NH_2).OH$.

This was followed by the discovery, by the Bayer firm, that the replacement of the azo-linkage in II by the peptide (—NH—CO—) linkage gave rise to a colourless substance also possessing trypanocidal properties.

It was known from work on dyes that the substantive character to cotton was favoured by the presence of chains of aminobenzoyl groups, and, by the introduction of such groups into the *s*-carbamide of an aminobenzoylnaphthylaminepolysulphonic acid, the important trypanocidal agent Bayer 205 (III) was evolved.

(III) $C_{10}H_4(SO_3H)_2NH.CO.C_6H_3(CH_3).NH.CO.C_6H_4.NH.CO.NH.C_6H_4.CO.NH.C_6H_3(CH_3).CO.NH.C_{10}H_4(SO_3H)_2.$

As Balaban and King [1927] point out, it is clear that the discovery of Bayer 205 arose from the recognition of the fact that it is possible to prepare colourless substances of substantive properties which, being of similar molecular build to trypanocidal dyes, also exhibit trypanocidal properties.

The next important step was taken by Balaban and King [1927], who started with six readily obtainable naphthylaminedisulphonic acids and from these prepared the *m*-aminobenzoyl and the *m'*-aminobenzoyl-*m*-aminobenzoyl derivatives. They then formed the *s*-carbamides of these derivatives by phosgenation. The *s*-carbamide of the original naphthylaminedisulphonic acid will be referred to as the first *s*-carbamide derivative; that of the *m*-aminobenzoyl derivative as the second *s*-carbamide derivative and that of the *m'*-aminobenzoyl-*m*-aminobenzoyl derivative as the third *s*-carbamide derivative.

Thus:

$NH_2.R \rightarrow R.NH.CO.NH.R$
First *s*-carbamide derivative

$NH_2.C_6H_4.CO.NH.R \rightarrow R.NH.CO.C_6H_4.NH.CO.NH.C_6H_4.CO.NH.R$
m-aminobenzoyl derivative — Second *s*-carbamide derivative

$NH_2.C_6H_4.CO.NH.C_6H_4.CO.NH.R \rightarrow R.NH.CO.C_6H_4.NH.CO.C_6H_4.NH.CO.NH.C_6H_4.CO.NH.C_6H_4.CO.NH.R$
m'-aminobenzoyl-*m*-aminobenzoyl derivative — Third *s*-carbamide derivative.

Balaban and King found, on examining the trypanocidal properties of the aminobenzoyl derivatives and of the first, second and third *s*-carbamides of the six naphthylaminedisulphonic acids, that trypanocidal activity only occurred among the second and third *s*-carbamides, *i.e.* at the *s*-carbamide stage of combination of the aminobenzoyl and aminobenzoyl-aminobenzoyl derivatives. Bayer 205, it is to be noted, is a third *s*-carbamide derivative. Following this, these workers then showed, on examining the substantive properties of these substances to cotton, that a pronounced maximum of substantivity occurred at the *s*-carbamide stage of combination and was greater with the third *s*-carbamides than with the second. Thus there appeared to be a definite parallelism between the substantive properties of these carbamides to cotton and their trypanocidal activity. Bayer 205 was also shown to be substantive to cotton.

Turning now to fumarase and knowing that trypan-blue, trypan-red and Bayer 205 were toxic to the enzyme, it became of interest to see whether the *s*-carbamides investigated by Balaban and King had any effect upon the

enzyme. Experiment has shown that toxicity only appears at the second *s*-carbamide stage of combination and that the third *s*-carbamides are highly toxic. The effect, moreover, is specific to fumarase, for urease is unaffected by these derivatives.

Method.

The method adopted throughout this work is that described fully in a previous communication [Quastel, 1931]. It consists, briefly, in mixing a preparation of fumarase, obtained from brain, with phosphate buffer solution, p_H 7·4, and the substance under investigation and in incubating the mixture for 30 min. or 60 min. at 45°. Sodium fumarate solution is then added to the mixture to make a final concentration of 0·08 *M*. Incubation is allowed to proceed for 2 hours at 45°, and the *l*-malate produced is estimated polarimetrically. From a comparison of the *l*-malate produced in presence of the toxic substance with that formed in its absence, an estimate of the inhibiting effect of the toxic agent is obtained. Details of the estimation of the *l*-malate will be found in the previous communication.

The effects of trypan-blue and trypan-red.

To a mixture of 2 cc. fumarase preparation and 1 cc. *M*/5 phosphate buffer, p_H 7·4, was added 1 cc. trypan-blue solution or 1 cc. trypan-red solution, to give a concentration of 1/4000. The mixture was incubated for 30 min. at 45°, and then 1 cc. 0·4 *M* sodium fumarate solution was added. Incubation was carried out for 2 hours at 45°. A mixture of 1 cc. glacial acetic acid and 10 cc. 14·2 % ammonium molybdate solution was then added, and the solution shaken with a little decolorising charcoal and filtered. The filtrate was examined polarimetrically in a 1 dm. tube, compared with a control experiment in which no dyestuff was present, and the amount of inhibition calculated.

The experiment was repeated with various concentrations of the dyes and the results are recorded in Table II. It will be seen that trypan-blue is more toxic than trypan-red but that both are toxic at concentrations of 1/16,000.

The effects of naphthylaminedisulphonic acids.

The following six acids were used:

Freund's acid: 1-naphthylamine-3 : 6-disulphonic acid.
Amino-G-acid: 2-naphthylamine-6 : 8-disulphonic acid.
C-acid: 2-naphthylamine-4 : 8-disulphonic acid.
Amino-J-acid: 2-naphthylamine-5 : 7-disulphonic acid.
H-acid: 8-hydroxy-1-naphthylamine-3 : 6-disulphonic acid.
2R-acid: 8-hydroxy-2-naphthylamine-3 : 6-disulphonic acid.

Experiments were carried out, as described for trypan-blue and trypan-red, but the incubation period prior to the addition of fumarate was one hour and in the estimation of the malic acid it was unnecessary to shake with charcoal.

Results are noted in Table I from which it will be seen that these acids at a concentration of 1/4000 have little or no toxic action on fumarase.

Table I. *Percentage inhibitions of fumarase activity by naphthylaminedisulphonic acids and their* s-*carbamide derivatives. Concentration* = 1/4000.

	Freund's acid	Amino-G-acid	C-acid	Amino-J-acid	H-acid	2R-acid
Free acid	1	0	0	0	3	2
1st *s*-carbamide	—	—	3	4	—	5
2nd *s*-carbamide	5	32	17	19	8	95
3rd *s*-carbamide	83	90	94	95	32	95

The effects of s-*carbamides of naphthylaminedisulphonic acids.*

Specimens of these substances were kindly supplied by Dr H. King. The first *s*-carbamides of Freund's acid, amino-G-acid, and H-acid were not available and were not tested.

Experiments were carried out exactly as with the naphthylaminedisulphonic acids and the complete set of results is given in Table I.

The most striking result is that, whereas the first *s*-carbamide derivatives are practically inert, the third *s*-carbamide derivatives are highly toxic.

There is a great difference in toxicity between the carbamide derivatives of H-acid and 2R-acid, the only chemical difference between these acids being that the former is a 1-naphthylamine derivative and the latter a 2-naphthylamine derivative. The second *s*-carbamide of 2R-acid is as toxic as any of the third *s*-carbamide derivatives of the acids investigated, and far more so than the other second *s*-carbamide derivatives. It would seem that the extra toxicity of 2R-acid is determined by the presence of the hydroxyl group in the naphthalene nucleus and this is supported by the fact that trypan-blue (which contains such a group) is more toxic than trypan-red (from which the hydroxyl group is absent). H-acid, however, which also contains the hydroxyl group in the naphthalene nucleus, forms *s*-carbamides which are not only less toxic than those from 2R-acid but less toxic than those from the non-hydroxylated naphthylaminedisulphonic acids. It is impossible, therefore, to attribute any increased toxicity solely to the hydroxyl group.

It is, however, quite clear that toxicity to the enzyme becomes apparent only at the second *s*-carbamide stage of combination and is most marked at the third *s*-carbamide stage. (Bayer 205 which is a third *s*-carbamide derivative gives a 68 % inhibition of fumarase at a concentration of 1/4000.) Now this is precisely the result obtained by Balaban and King [1927] who found that trypanocidal activity became apparent only at the second *s*-carbamide stage of combination and was most marked at the third *s*-carbamide stage.

It must not be supposed that the toxicity of the higher *s*-carbamides to fumarase is an explanation of their trypanocidal activity, for Balaban and King found that the derivatives of Freund's acid and 2R-acid have no trypanocidal action. It is evident, however, that there must be some structure common to the trypanosome and to fumarase which accounts for the parallelism which

exists between fumarase toxicity and the trypanocidal action of the second or third *s*-carbamides.

Variation in toxicity with concentration of s*-carbamides.*

The experiments described above were repeated with the more toxic *s*-carbamides at various concentrations, the incubation period prior to the addition of fumarate being 30 min. The results are recorded in Table II. The most important result emerging from this Table is that the *s*-carbamide compounds which retain a high toxicity at considerable dilution are all derived from 2-naphthylamine structures. Derivatives of the 1-naphthylamine compounds (Freund's acid and H-acid) have relatively little toxicity at high dilution.

Table II. *Percentage inhibition of fumarase activity by by* s*-carbamides* etc. *at various concentrations.*

	Concentration		
	1/4000	1/8000	1/16,000
Trypan-blue	100	100	92
Trypan-red	74	43	21
3rd *s*-carbamide of Freund's acid	76	52	14
3rd *s*-carbamide of H-acid	29	11	6
2nd *s*-carbamide of amino-G-acid	16	11	3
3rd *s*-carbamide of amino-G-acid	87	78	60
3rd *s*-carbamide of C-acid	97	95	85
3rd *s*-carbamide of amino-J-acid	91	79	45
2nd *s*-carbamide of 2R-acid	93	87	75
3rd *s*-carbamide of 2R-acid	90	80	53

Substantive properties and toxic action.

Balaban and King suggested from their experiments that there is a rough parallelism between the substantivity to cotton and the trypanocidal action *in vivo*, in that the salient peaks of substantivity occurred at the *s*-carbamide stage of combination. They were only able to determine the substantivity of the derivatives of H- and 2R-acids, but since Bayer 205 was also found to be substantive to cotton, it seems likely that the phenomenon holds also for the nonhydroxylated derivatives.

It is obvious that there is also a parallelism between substantivity to cotton and toxicity to fumarase, in that the latter appears only among the second and third *s*-carbamides. The parallelism, however, must be very rough, for whereas the substantive power of the second carbamide of 2R-acid is $2\frac{1}{4}$ times that of the second carbamide of H-acid, the toxic power of the former on fumarase is about 12 times that of the latter.

It is significant in this connection that Congo red and benzopurpurin which are highly toxic to fumarase are substantive to cotton.

On the whole it would seem that the fumarase enzyme, which is very widespread biologically both in plant and animal tissues, must possess structures which are common to the cotton fibre and to the trypanosome and which are responsible for the specific combination or adsorption of the higher

s-carbamide derivatives of the naphthylaminedisulphonic acids. Other factors, specific for the type of biological material, doubtless account for the variations which are observed between the effects of these derivatives on the different biological types.

It is of particular interest that there should be an enzyme to which these trypanocidal agents exhibit a specific toxicity (urease, for instance, is unaffected by them) and the elucidation of the factors involved should be of material assistance in the development of this branch of chemotherapy.

Protection by fumarate.

It has been shown [Quastel, 1931] that fumarase is "protected" by its substrate, fumarate, from such toxic dyestuffs as Congo red and methyl violet. This holds also for the *s*-carbamide derivatives.

To 2 cc. fumarase preparation were added 1 cc. *M*/5 phosphate buffer, p_H 7·4, 1 cc. water, and 1 cc. 0·4 *M* sodium fumarate solution. After incubation at 45° for 2 hours the rotation was 1·12°.

To 2 cc. fumarase preparation were added 1 cc. *M*/5 phosphate buffer, p_H 7·4, 1 cc. 1/1000 solution of the third *s*-carbamide derivative of 2R-acid, and 1 cc. 0·4 *M* sodium fumarate solution. After incubation for 2 hours the rotation was 1·03°. (Inhibition = 8 %.)

To 2 cc. fumarase preparation were added 1 cc. *M*/5 phosphate buffer, p_H 7·4, and 1 cc. of 1/1000 solution of the third *s*-carbamide derivative of 2R-acid. The mixture was incubated at 45° for 30 min. and then 1 cc. 0·4 *M* sodium fumarate solution was added. After incubation at 45° for 2 hours the rotation was 0·11°. (Inhibition = 90 %.)

The experiment shows that incubation with the *s*-carbamide for 30 min. prior to the addition of fumarate gave an inhibition of 90 %, whereas when the derivative was added together with the fumarate the inhibition was only 8 %.

This holds also for other *s*-carbamide derivatives.

Effects of proteins on the toxic action of s-carbamides.

As occurs in the case of the toxic dyestuffs, proteins exert a marked protective action against the *s*-carbamides.

This is indicated in Table III where the effects of addition of guinea-pig serum at different concentrations to fumarase are noted.

Table III. *Percentage inhibitions of fumarase by s-carbamides (1/5000) in presence of serum.*

Concentration of serum	3rd carbamide of amino-J-acid	3rd carbamide of 2R-acid	Bayer 205
1/25	36	40	20
1/50	81	70	33
1/100	89	84	37
1/200	92	91	43
0	92	92	43

A typical experiment was as follows. To 2 cc. fumarase preparation were added 1 cc. *M*/5 phosphate buffer, 1 cc. of diluted guinea-pig serum, and 1 cc.

of 1/1000 solution of the *s*-carbamide. This was allowed to incubate at 45° for 30 min. and then 1 cc. 0·4 *M* sodium fumarate solution was added. After further incubation at 45° for 2 hours the rotation was determined and the percentage inhibition calculated.

There is little doubt that, as in the case of the dyestuffs, the protective action of the protein is due to a combination of the protein with the *s*-carbamide derivative, resulting in a diminution of the concentration of the free derivative and hence in a reduced inhibition of fumarase activity.

Summary.

1. Trypan-blue, trypan-red, and Bayer 205 are toxic to fumarase.

2. The toxicity of six naphthylaminedisulphonic acids and their *s*-carbamide derivatives on fumarase has been investigated. It is shown that the free acids and their first *s*-carbamide derivatives are inert. Toxicity begins to be apparent with the second carbamide derivatives (*s*-carbamide of *m*-aminobenzoylnaphthylaminedisulphonic acid) and is very marked with third carbamide derivatives (*s*-carbamide of *m'*-aminobenzoyl-*m*-aminobenzoylnaphthylaminedisulphonic acid). Attention is drawn to the parallelism which exists between this effect and trypanocidal action which begins only at the second *s*-carbamide stage of combination and is most marked at the third *s*-carbamide stage. The parallelism with the substantive properties of these derivatives to cotton is also discussed, and it is shown that there must be some structure in common between the fumarase enzyme, cotton fibre and the trypanosome which makes for specific combination or adsorption with the second and third *s*-carbamide derivatives of the naphthylaminedisulphonic acids. These derivatives are not toxic to urease.

3. Derivatives of the 2-naphthylaminedisulphonic acids are much more toxic to fumarase than those of the 1-naphthylaminedisulphonic acids.

4. Fumarate "protects" the enzyme, fumarase, from the toxic action of these derivatives.

5. The presence of proteins diminishes or eliminates the toxic action of these derivatives.

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CXXVIII. THE RELATION OF GLYCOSURIA TO GLYCAEMIA AND THE DETERMINATION OF THE RENAL THRESHOLD FOR GLUCOSE.

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Introduction.

Glycosuria, as the most obtrusive finding in diabetes mellitus, has, since its association with this disease was established, aroused considerable interest both as to its production and as to its significance. Prior to the day of Claude Bernard the presence of sugar in the blood was regarded as denoting an abnormal and definitely pathological condition, and glycosuria was considered to be its attendant, and equally pathological, manifestation. His demonstration that glycaemia was of normal occurrence, and that hyperglycaemia was productive of glycosuria, was the first step towards the modern ideas on the subject, and, in addition, it provided the data upon which Bernard [1877] based his conception of the renal threshold as that level of sugar in the blood at which glycosuria appeared. Some years later the discovery by von Mering [1888] of phlorhidzin diabetes showed that glycosuria could exist apart from hyperglycaemia, but it was only with the realisation that there existed a benign clinical type of this disorder that the idea of the presence of a renal factor in all cases of glycosuria became generally accepted.

Apparently Bernard was of the opinion that when the sugar in the blood exceeded a certain level the kidney permitted sugar to "spill" over into the urine. A definite line could therefore be drawn between glycosuria and aglycosuria. This idea was universally accepted until it was attacked by Benedict, Osterberg and Neuwirth [1918]. They maintain that, in the urine of normal individuals, sugar is always present, and that, after carbohydrate meals, an increase in amount may be detected. They have suggested the term glycuresis for this phenomenon, and claim that there is no such thing as a definite threshold. Folin and Berglund [1922] have criticised this work on the ground that the carbohydrates present in normal urine are not of the nature of glucose, but are unusable carbohydrates which the body naturally excretes. Until glucose has been isolated from normal urine this conception of glycuresis must be considered unproven.

The work of Richards [1929] has produced very strong evidence in favour of the filtration-reabsorption theory of kidney function and considered along

with the recent work of Ni and Rehberg [1930] has thrown considerable light upon the nature of the renal threshold. Richards has demonstrated in frogs the presence of glucose in the glomerular filtrate and its absence from the urine. By studying the blood-sugar and the glycosuria in dogs after the intravenous administration of glucose, in conjunction with Rehberg's creatinine filtrate method of estimating the amount of reabsorbed fluid, Ni and Rehberg have shown that as the blood-sugar rises the amount of glucose reabsorbed by the tubules rises also, but that this increase becomes less and less the higher the level attained by the blood-sugar, and that when the difference in concentration between the sugar in the blood and the sugar in the lumen of the tubules exceeds a certain limit, there is no further increase in reabsorption. It appears therefore that for every concentration of blood-sugar a definite amount of glucose per minute is filtered through the glomerulus, and that also for the same value a definite amount of glucose per minute is absorbed from the lumen of the tubules. Thus there will be one value of the blood-sugar at which the tubules just fail to absorb all the glucose filtered, and glycosuria will occur. This value will correspond to the renal threshold.

A peculiar phenomenon in connection with the threshold was first observed by Frank [1913] and has since been confirmed by many other investigators. Frank found that the level of the blood-sugar at which glycosuria appeared was not the same as that at which it disappeared. In his experience the former was usually the higher, but many cases have been reported in which the latter was the higher. Most observers have been inclined to accept this phenomenon as indicating the presence of two different normal thresholds, but Faber and Hansen [1923] in their review of the subject remark, "we cannot really regard the difference as a sign of change in the threshold; it is a phenomenon by itself which must be separated from the level of the threshold." And up to the present there has been no definite evidence to disprove this statement.

Thus it appears that the balance of evidence is in favour of a renal threshold, or thresholds, of a definite level, and that there is some indirect evidence as to the nature of the underlying mechanism.

Methods previously advocated for the determination of the renal threshold.

When considering the methods which have been recommended for the determination of the renal threshold, one must remember that some of these methods were evolved before much of the now commonplace knowledge of the behaviour of the blood-sugar was discovered, and in assaying any method it is essential to bear the following three facts in mind. First, that the blood-sugar varies considerably throughout the 24 hours according to the food intake; second, that the level of blood-sugar at which glycosuria appears usually differs from the level at which it disappears; and third, that in most,

if not all diabetics, during the night, the blood-sugar does not exhibit a uniform decrease but rises steadily in the early hours of the morning, and then decreases again slowly. This latter phenomenon, noted by Hatlehol [1924], has been confirmed by the writer.

The methods will now be described, and in the consideration of methods (*a*) and (*b*) the debt to the critical review of Faber and Hansen [1923] must be acknowledged.

(*a*) After the night's fast the blood-sugar is estimated, and the urine is tested for the presence or absence of sugar. This procedure is repeated several times during treatment, and from the data thus collected the threshold is adjudged to lie between certain values of the blood-sugar.

Certain workers [Williams and Humphreys, 1919] have based their conclusions upon the presence or absence of sugar in a 24-hour specimen, but in the light of the known variations of the blood-sugar the inaccuracy of such conclusions is obvious. The most accurate modification of the test is that in which the bladder is emptied after a night's fast, urine is passed again a short time later, and the blood-sugar estimated half-way between the two evacuations. From the data thus collected some knowledge of the relationship of the blood-sugar values to the presence or absence of glycosuria may be gathered, but it is obviously impossible by any modification of this test to avoid the discrepancies introduced by the two different thresholds. It is the usual experience with diabetics that as the patient reacts satisfactorily to treatment the value of the morning blood-sugar decreases, and there is a concomitant decrease, and possibly disappearance, of the glycosuria. In a case in which the threshold of appearance is higher than the threshold of disappearance, glycosuria may occur on one occasion and not on another with a blood-sugar of approximately the same value. Failure to take this fact into consideration would account for the common statement that, during treatment, there is a rise in value of the renal threshold.

It appears that this method, and the conclusions drawn from it, have little or no value.

(*b*) After the oral administration of a standard dose of glucose, the blood-sugar is estimated at frequent intervals, and the urine voided at frequent known intervals is tested for sugar. By noting the highest value of the blood-sugar both on the rise and on the fall of the curve, at which the urine is sugar-free, and similarly the minimum value at which glycosuria occurs, an approximate idea of the two thresholds may be obtained.

This method has been used extensively but is open to criticism on the score of the practical difficulties which prevent its accuracy. The frequency with which the bladder can be emptied is limited, and 10-minute specimens are as much as can be expected from an unpractised person. It will easily be seen that, under such natural restrictions, with a rapidly rising or falling blood-sugar curve, considerable error is inevitable, but that on a slowly falling curve a reasonable degree of accuracy can be attained.

Another criticism, which applies equally to all methods involving qualitative testing of the urine for sugar, is the question of the sensitivity of the reagent. The lower limit of sensitivity of Benedict's reagent is in the region of 0·09 % of glucose. Now, if the threshold is exceeded just before a urination the sugar-containing urine then passed will be diluted by the urine already in the urinary tract, and the resulting concentration of sugar may be too small to be detected by the reagent. In cases of renal glycosuria where the concentration of sugar excreted is very low this is a point of considerable importance.

A method similar in principle, and limitations, is that in which the glycosuriac is starved until he is sugar-free, blood-sugar estimations being performed the while. As the slope of such a blood-sugar curve is very gradual, provided one does not meet the nocturnal rise of blood-sugar, one might expect to get a moderately accurate idea of the situation of the threshold of disappearance, but according to Faber and Hansen [1923] this point appears to be lower after starvation than after administration of glucose. Further information is wanted on this point before the factor of experimental error either in this latter method or their control method can be ruled out.

(*c*) On different days graded doses of glucose are given to the patient, the blood-sugar estimated frequently in each experiment, the resulting curve charted, the urine passed frequently and tested for sugar. Curves of varying heights are obtained. The peaks on the lowest curve with glycosuria and the highest curve without are noted and the threshold is said to lie between these two points. The authors of this method, Faber and Hansen [1923], point out that it is essential to estimate the blood-sugar frequently in order to hit the peak of the curve, and also, that it is equally essential to obtain frequent urine specimens as the concentration of the sugar passed, in those experiments near the border line of glycosuria, is so small that dilution renders impossible its detection by the reagent. They claim that it is quite possible, with practice, so to judge the dose of sugar that the peaks of neighbouring curves may be made to lie very close to each other. The reason behind the evolution of this method is to be found in the statement by the above authors quoted previously, namely, that two thresholds do not exist, there being but one threshold which has the apparent phenomenon of the double threshold superimposed upon it. From the results given later two separate thresholds do appear to exist, and, in that case, their figures will correspond to determinations of the threshold of appearance.

(*d*) By a modification of Ambard's formula, Chabanier, Lebert and Lobo-Onell [1929] have produced an equation expressing a theoretical relationship between blood- and urine-sugars and the renal threshold. Their results and conclusions are so much at variance with the observations on this subject that they need not be discussed further.

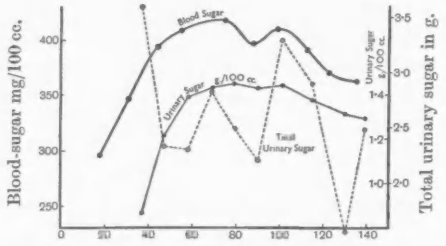
It will be seen that none of the methods above described attains to any high degree of accuracy, and that they are all equally inapplicable in the case of a patient who is never sugar-free. The second method described probably

gives the least distorted results of all, but its limited accuracy has always left doubts as to the validity of conclusions based upon its results.

New method.

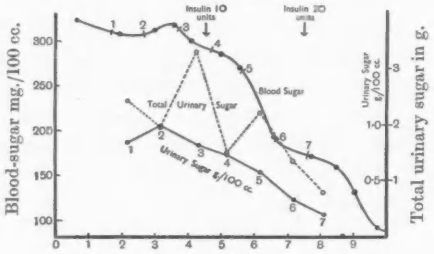
The theoretical basis.

The question of the renal threshold is so intimately bound up with the problem of the relationship of glycosuria to glycaemia that they cannot be discussed apart. These various relationships are well brought out by the data obtained from a sugar tolerance curve, in which frequent observations of blood-sugar and urine-sugar values are made at definite intervals, and then charted on the same time scale. It is immediately obvious that, whilst there is no apparent correspondence between the glycaemia and the total amount of urinary sugar, there is a definite parallelism between the concentration of sugar in the blood and the concentration of sugar in the urine (Figs. 1 and 2).



Time in minutes after administration of 50 g. glucose

Fig. 1.



Time in hours

Fig. 2.

This correspondence is so close that it suggests a proportionate relationship between the two variables. If now the figures for the concentration of glucose in the urine are plotted along the abscissa against points on the ordinate denoting the preceding blood-sugar values at those points in time midway between two consecutive urinations, points are obtained lying along a straight line which cuts the ordinate at some point above zero (Figs. 3, 5 and 7). From

such a graph it is possible to read off the blood-sugar values corresponding to any given concentration of urinary sugar.

Now when the blood-sugar is below and until it attains the renal threshold, no sugar appears in the urine. When the threshold is just reached, sugar is still absent from the urine and immediately it is passed a definite concentration of sugar appears. On such a graph, therefore, the blood-sugar value corresponding to zero on the abscissa is the renal threshold value, and this is the point where the straight line cuts the ordinate.

It will be noted on reference to Figs. 5 and 7 that two lines are obtained. One marked "rise" is drawn through the points obtained whilst the blood-sugar curve is rising, and on the other marked "fall" through those taken during the fall of the curve. This point is well brought out when the data from two consecutive tolerance curves are taken. The two different threshold values obtained correspond respectively to the threshold of appearance and the threshold of disappearance. In Fig. 3 the values charted being taken

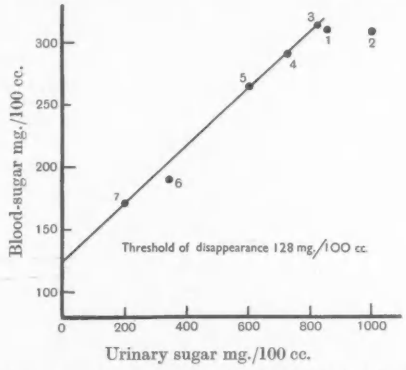


Fig. 3. (Linear graph corresponding to Fig. 2.) Note that the values taken before the fall was established fall off the line.

exclusively from a fall curve (Fig. 2), only the threshold of disappearance is shown. In all cases in which two lines are obtained it will be found that these are never continuous; they are separated by an indeterminate zone corresponding to the higher blood-sugar values found round the peak of the tolerance curve, and in this zone various points interpolated from these values fall. Only in those cases in which the peak is flattened into a plateau can a sufficient number of points be obtained to give some idea of the happenings in this area. From the data shown in Fig. 8 it will be seen (Fig. 9) that these points tend to fall on a smooth curve, in this case continuous with the fall line, which, if continued, would make a sharp angle with the rise line. Consideration of these and other figures suggests that the junction between the two lines is effected, not by a single, but by an S-shaped curve, and that this is not necessarily enclosed in its entirety by the two lines.

From the linear graph it is possible to deduce a formula by which the renal threshold may be obtained. Firstly, it will be seen that for equal variations in blood-sugar values there are equivalent variations in the figures for the concentration of urinary sugar. Secondly, that the ratio between the height of the blood-sugar above the renal threshold and the corresponding urinary sugar is constant.

Let B_1, B_2, B_3 = value of the blood-sugar in mg./100 cc.

Let W_1, W_2, W_3 = value of the urinary sugar in mg./100 cc.

Let x = renal threshold.

Then $B - x$ = head of blood-sugar above the renal threshold.

Now $$\frac{B_1 - x}{W_1} = \frac{B_2 - x}{W_2} = \frac{B_3 - x}{W_3} = K,$$

and when $$B_1 - B_2 = B_2 - B_3,$$

then $$W_1 - W_2 = W_2 - W_3.$$

Consider a blood-sugar value which is $(B_1 - B_2)$ mg./100 cc. above x, *i.e.* $(B_1 - B_2) + x$.

When the blood-sugar falls from B_1 to B_2 the concentration of sugar in the urine falls from W_1 to W_2, *i.e.* $(W_1 - W_2)$ mg./100 cc. Therefore the blood-sugar value $(B_1 - B_2) + x$ corresponds to a urinary sugar concentration $W_1 - W_2$.

Therefore $$\frac{[(B_1 - B_2) + x] - x}{W_1 - W_2} = K = \frac{B_1 - x}{W_1},$$

$$\frac{B_1 - B_2}{W_1 - W_2} = \frac{B_1 - x}{W_1},$$

whence $$x = \frac{W_1 B_2 - W_2 B_1}{W_1 - W_2}.$$

Thus it is theoretically possible to find the renal threshold value from two consecutive figures expressing the urine-sugar in mg./100 cc., and the two corresponding blood-sugar values.

The technique of the method.

The patient is starved for 18 hours before the test is to be performed. During this time he is allowed water *ad lib.*, and at the 14th hour, after emptying the bladder, he is given 1 pint of water. At the 16th hour the bladder is emptied again, another pint of water taken, and at 17½ hours this is repeated. By this means the mucous membrane of the bladder is washed free from any adherent sugar. At the 18th hour the resting blood-sugar is determined, the bladder emptied, and 50 g. of glucose given by mouth. At 10-minute intervals further blood and urine samples are taken, and after each urination the patient is given 300 cc. of water to drink. Each specimen of urine is tested for sugar and only those specimens saved which give a positive reaction.

The collection of the blood offers no difficulty, but various points must be attended to in the collection of the urine. The large amount of water taken

not only removes the discrepancy referred to later, but provides sufficient fluid for the frequent micturitions necessary. Under its influence it will be found quite easy to obtain 10-minute samples from men, but in women it is essential to pass a catheter. The most convenient way of doing this is by means of a male gum elastic catheter complete with a well-fitting spigot. At the beginning of the test this is passed and secured in position by means of tapes and adhesive plaster. All that it is then necessary to do in order to collect a specimen is to remove the spigot and press on the anterior abdominal wall. When the urine ceases to flow the pressure is maintained and the catheter withdrawn slightly to ensure that there is no retention due to the tip projecting too far into the bladder. The point to be insisted on in both sexes is that the bladder be emptied as completely as possible at each urination.

The question now arises as to the efficiency with which the urinary tract can be evacuated and the error introduced into the glucose concentration figures by that degree of urinary retention which is inevitable. Obviously it is impossible to empty the ureter and the pelvis of the kidney, and the urine contained in them will appear in the next specimen. This part of the tract usually contains about 5 cc. of fluid. The diuresis induced during the investigation is of the order of 100 to 200 cc. per 10 minutes. If the blood-sugar values are rising or falling slowly the concentration of urinary sugar rises or falls by small amounts in consecutive specimens, and the correspondence between the two is practically perfect. If the variations are more marked the corresponding error becomes more pronounced, but this can be overcome to some extent by increasing the diuresis. For this reason it is always more important to have a marked diuresis on the rise, which is rapid, than on the fall of the blood-sugar curve, which is slow. The correspondence demonstrated in the ensuing results shows that there is here no serious source of error.

The first specimen, after the renal threshold has been passed on the rise of the curve, will possibly be diluted by the urine passed immediately previous to this. For this reason this specimen may be misleading and in that case, when the figures relative to it are charted, a discrepancy will be revealed. This specimen should not however be discarded, for, as pointed out in the section discussing the accuracy of the method, sugar may have been present in the specimen previous to this but have been diluted beyond the limits of detection of the qualitative reagent, and in that case the first sample giving a positive reaction will contain its full complement of sugar. On the fall a similar discrepancy may arise, for whilst the specimen passed immediately after the fall below the threshold may contain a sufficient concentration of sugar to give a qualitative reaction, this may still fall short of the maximum concentration possible.

We have seen that when the curve is in the region of the peak, values are obtained which on charting give points falling in the indeterminate zone between the two lines. These points are serious sources of error if included in the calculation of the threshold. Therefore, values should always be taken as

near each threshold as possible. Consecutive specimens after the appearance of sugar on the rise fulfil this condition, but in order to obtain similar specimens on the fall one is driven, if the curve is at all prolonged, to forming an opinion, from clinical data, as to the time when the fall will be well established. If the urine passed before the test is commenced contains sugar, it is wise to assume that the curve will take a considerable time to return to the normal level. All the data will be obtained, and the determination considerably shortened, if the blood-sugar is first depressed by the injection of 15 units of insulin and then raised by the subsequent administration of glucose.

It is hardly necessary to point out that if the blood-sugar curve is falling slowly specimens may be collected at longer intervals than 10 minutes, and quite accurate results obtained.

Theoretically, each threshold may be determined from two urine and two blood samples, but in practice it is more accurate to take three specimens of each, to chart them, draw the line which corresponds most closely to the three points, and by reading off two sets of values from this line apply the equation and obtain the threshold.

To obtain the following data MacLean's method was used for the estimation of the blood-sugar, and for the urinary sugar Benedict's method in the early cases and the polarimeter in the later. There have been many criticisms of the estimation of sugar by the polarimeter. The instrument used was a Hilger M 2 with direct vision spectroscope attachment and the light was provided by a mercury vapour lamp. The specific rotation of glucose was taken as $[\alpha]_D + 52{\cdot}5°$ and the readings were obtained for the mercury green line corrected to α_D by dividing with the factor 1·18. The error involved in the reading amounted to 0·02°. With the diuresis obtained the specimens were clear and it was found that such a urine, containing no glucose, showed no rotation.

Lastly it must be emphasised that to obtain good results the conditions laid down above must be adhered to strictly, as under other conditions discrepancies may occur.

The accuracy of the method.

In discussing the accuracy of this method there are two distinct considerations to be taken into account. One is the accuracy of the methods used in the determination of the necessary data, and the second is the accuracy of the method itself relative to these data.

The accuracy attainable by the various methods used in the determination of the blood-sugar is still under dispute and will not be discussed. As regards the urine-sugar, the majority of urines analysed being of low concentration in sugar, Benedict's method did not give an end-point sufficiently sharp, but owing to the conditions of the experiment accurate readings in practically all the specimens containing their full complement of sugar could be obtained on the polarimeter.

With regard to the second consideration, the whole question turns upon the variation in position of the line allowed by the points in the linear graph. The conditions necessary for the obtaining of good results had been laid down when the experiment, set forth in Figs. 4 and 5, was performed. It will be

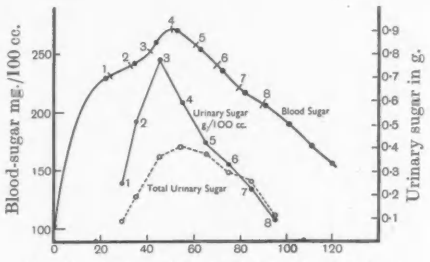


Fig. 4.

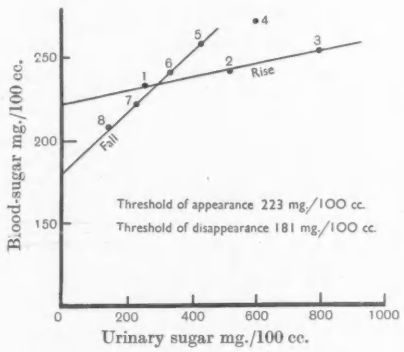


Fig. 5. Linear graph corresponding to Fig. 4.

seen (Fig. 5) that on the fall the line cuts each point and that no variation is possible. The threshold of disappearance is definitely 181 mg./100 cc. On the rise a variation is allowed and the maximum value of this is 2 mg., that is, the threshold of appearance lies between 221 mg./100 cc. and 223 mg./100 cc. Greater variations do occur, but even on the rapidly rising curve of a case of renal glycosuria these should not exceed 6 or 7 mg. of the total blood-sugar, whilst on a slowly moving curve practically no latitude should be allowed.

One of the advantages of the method is that it is not limited by the sensitivity of qualitative reagents. This is well illustrated by case 6 and the case used for Figs. 4 and 5. Consider, in the former, the rising blood-sugar curve. By the above method the threshold of appearance is 250 mg./100 cc. The 250 mark is crossed at 23 mins., but at 28 mins. a urine giving a negative

Benedict's reaction is passed. The preceding specimen, also with a negative Benedict's reaction, was passed at 17 mins. From the blood-sugar curve and linear graph the following data can be adduced.

At 28 mins.	the blood-sugar is	268 mg./100 cc.	corresponding to	200 mg./100 cc.	urinary sugar.
„ 27	„	266	„	170	„
„ 26	„	262	„	140	„
„ 25	„	258	„	90	„
„ 24	„	254	„	50	„
„ 23	„	250	„	0	„

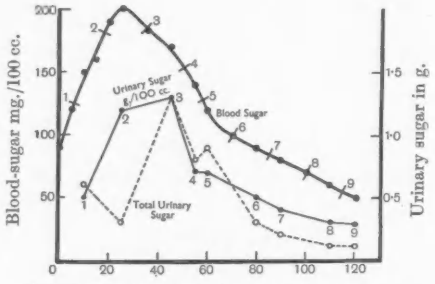


Fig. 6. Case of renal glycosuria. Figures taken from *Acta Med. Scand.* Supp. XXVII, p. 209 (Malmros).

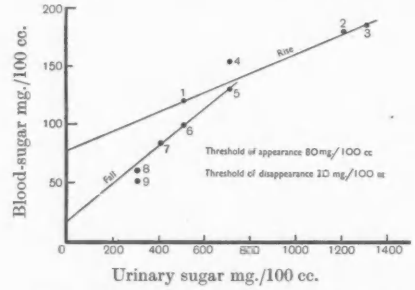


Fig. 7. Linear graph corresponding to Fig. 6.

The average content of sugar in the urine over the 5 mins. is 130 mg./100 cc., but this is diluted by urine passed in the preceding 6 mins., and, if we assume that the diuresis is approximately the same over these 11 mins., then the average concentration of urinary sugar passed at 28 mins. is 60 mg./100 cc., which is undetectable by Benedict's reagent.

In all cases in which the threshold has been determined by the above method, and in which a rough idea of the threshold could be directly obtained, no discrepancy has been found.

It may then be concluded that even with the limited accuracy of the means of estimation at present available, the method gives practically perfect results.

Results.

In Table I are given the results in 20 cases. Eleven of these have been arrived at from estimations done by the writer, and nine from figures taken from the literature. It is worthy of comment that in few instances, amongst

Table I.

Own cases				Cases from literature			
No.	Case	Threshold of appearance mg./100 cc.	Threshold of disappearance mg./100 cc.	No.	Reference	Threshold of appearance mg./100 cc.	Threshold of disappearance mg./100 cc.
1	D.W.	—	128	1	Malmros [1928], p. 209	79	20
2	E.F.	—	232	2	,, p. 241	30	103
3	S.D.	—	266	3	,, p. 210	—	70
4	H.W.	206	200	4	,, p. 233	86	104
5	E.P.	200	195	5	Hatlehol [1924], p. 236	110	142
6	G.L.	250	304	6	,, p. 237	234	224
7	I.H.	160	182	7	Bailey [1919], Case 2	73	50
8	H.P.	250	235	8	,, Case 7	232	192
9	H.M.	223	181	9	Goldblatt and Ellis [1931], Case 3, glucose	129	170
10	M.J.	194	175				
11	A.J.	152	144				

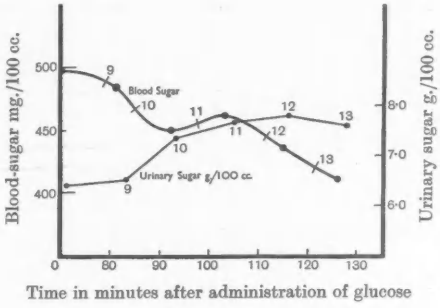


Fig. 8.

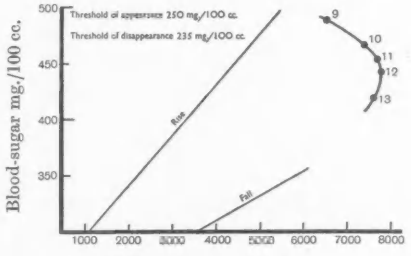


Fig. 9. Linear graph corresponding to Fig. 8. The "rise" and "fall" lines are taken from a previous experiment.

the multitude of figures recorded for glucose tolerance curves, were the corresponding percentages of urinary sugar noted, and in some cases in which they were noted the experiments were unsuitable for calculation by reason of their complicated nature. Perfect results are not to be expected from such figures, as the exact time relationships between the urine and blood samples are not recorded.

It will be noted that in no example are the two thresholds at the same level, although in many instances they lie very close together. This latter

Table II.

	Blood		Urine			
Case	Time in mins.	Sugar mg./100 cc.	Time in mins.	Volume cc.	Sugar %	Total sugar g.
6	0	189	—	—	—	—
Diabetic	19	236	—	—	—	—
	32	282	28	62	0	0
	49	332	47	78	0·538	0·429
	59	350	57	51	0·870	0·444
	75	374	70	78	1·144	0·891
	88	374	85	117	1·238	1·44
	179	349	175	650	0·850	5·52
	190	337	187	120	0·650	0·78
	202	318	199	76	0·385	0·293
	218	308	215	67	0	0
	228	299	—	—	—	—
10	0	—	0	—	0	0
Diabetic	12	182	14	—	0	0
	24	200	27	—	0	0
	35	216	39	100	0·105	0·105
	46	237	50	83	0·298	0·248
	57	268	60	73	0·52	0·285
	68	288	71	84	0·73	0·6
	78	310	81	82	0·86	0·71
	87	322	90	82	0·99	0·81
	98	330	100	91	1·06	0·96
	108	306	112	120	0·96	1·15
	119	278	121	115	0·72	0·83
	131	253	133	120	0·56	0·67
	142	243	144	112	0·46	0·51
9	0	103	7	—	0	0
Diabetic	12	208	18	65	0	0
	22	231	29	35	0·250	0·087
	34	244	35	36	0·515	0·196
	43	260	45	47	0·780	0·364
	52	271	54	63	0·598	0·405
	62	254	65	88	0·427	0·375
	72	237	74	90	0·330	0·298
	82	218	85	113	0·226	0·256
	91	207	95	109	0·097	0·105
	101	191	106	93	0	0
	111	172	115	70	0	0
	120	157	—	—	—	—
11	0	103	—	—	—	—
Renal	14	140	10	24	0	0
glycosuriac	22	177	27	15	0·12	0·028
	40	189	35	14	0·525	0·077
	53	176	49	43	0·25	0·108
	66	160	62	86	0·13	0·111
	82	142	78	170	0·065	0·110
	99	125	—	—	0	0
	119	114	—	—	—	—

apparent correspondence is deceptive, for in every case the two lines giving the thresholds showed distinctly different inclinations.

In Table II the figures from four of the author's experiments are set forth. It will be noticed that the diuresis is not marked in any of them, and we may say at once, that the minimum diuresis required for correspondence varies within wide limits for different individuals.

Discussion.

The primary observation upon which the preceding work is based is that, whilst, under the above conditions, there is a relationship between the concentration of sugar in the urine and the concentration of sugar in the blood, the total amount of sugar in the urine bears no relation to the glycaemia but rather reflects the water output. The important corollary of this observation is that the concentration of sugar in the urine is independent of the diuresis. Now the sugar in the urine is derived from the sugar in the blood, and yet it is impossible to lower the blood-sugar level by creating a diuresis and transferring large amounts of sugar from the blood to the urine. The concentration of sugar in the blood remains steady, the dependent concentration of urinary sugar is unaltered, the total amount of sugar in the urine varies within wide limits. Point is lent to this statement when it is remembered that whilst a patient with a blood-sugar of 300 mg./100 cc. may by diuresis be made to lose 10, 15 or 20 g. of sugar each hour, yet the total amount of sugar in his blood lies between 12 to 15 g. There can only be one conclusion from this phenomenon, and that is that as the body must be supplying sugar to the blood to keep the concentration constant, then the blood-sugar is being maintained at that concentration in spite of all influences to the contrary. Regarded from this point of view the sugar tolerance curve takes on an importance greater than that of a passive expression of the quantity of sugar in the blood awaiting storage, and the high blood-sugar of the diabetic acquires a new significance. Each level of glycaemia must be looked upon as a dynamic rather than a static condition; each as the optimum level for the needs of the body under the particular combination of circumstances then obtaining.

In explanation of the above observations, and the relationships deduced from them, the following theory is offered.

It is a well-known fact that insulin can be demonstrated in the body in tissues other than the pancreas, and that, in a diabetic who has died in coma, insulin may be recovered in surprising quantities from other structures than that organ. It is assumed that in life an insulin factor (I.F.) is present in all living tissues. Under a particular set of circumstances the body requires a definite amount of energy per unit of time, and of this energy each tissue requires its own particular quota. To produce this energy per unit of time a certain amount of carbohydrate must be utilised at a certain rate. Let it be assumed that the utilisation of glucose in the organism for the production of energy involves two consecutive reactions, (*a*) the formation, by the inter-

action of the glucose and tissue I.F., of an insulin-glucose compound, and (*b*) the breakdown of this compound with the simultaneous oxidation of the glucose and the production of energy. It is suggested that these reactions conform with the law of mass action and that the concentration of available oxygen required for reaction (*b*) is constant. In that case the initial velocity of reaction (*b*) will be proportional to the concentration of the insulin-glucose compound which itself is being formed, according to reaction (*a*), at a rate proportional to the product of the concentrations of insulin and glucose. Now under resting conditions the demand of the body for production of energy may be regarded as practically constant. If this demand is satisfied by the degradation of the insulin-glucose compound, the concentration in the tissues of the latter must also remain constant. It follows then that the formation of the insulin-glucose compound is proceeding with unvarying velocity, and therefore that the product of the concentrations of I.F. and glucose, which determine this velocity, is a constant quantity. Thus, when the production of energy is constant, if the concentration of insulin be increased, then a fall in the concentration of the blood-sugar is to be anticipated and *vice versa*.

The common observations of (1) the reduction in blood-sugar concentration following injection of insulin, and (2) the increasing blood-sugar concentration which accompanies the progressive restriction of insulin by disease, would follow as immediate corollaries of the above hypothesis. As a further example on this basis may be explained the results which follow administration of glucose.

It is well known that after the oral administration of glucose to a patient at rest there is a rise and fall in blood-sugar values, immediately preceded by a sharp rise and sharp fall in the respiratory quotient and the metabolic rate. The theory outlined above affords an explanation of these phenomena if the additional assumption be made that an increase in concentration of the blood-sugar does not cause an immediate decrease in that of the I.F. In that case the increase in concentration of the blood-sugar, subsequent to ingestion of glucose, will increase the velocity of reaction (*a*), and the resulting higher concentration of the insulin-glucose compound will bring in its train an increased rate of degradation and hence of energy production. The latter will manifest itself in a raised metabolic rate, the increased degradation in an approximation of the respiratory quotient to unity.

The limit of increase in these related phenomena will be determined by the concentration available in the tissues of I.F. and as this falls the respiratory quotient and metabolic rate will revert to resting values in spite of the persistence of a high blood-sugar. Since the product of the concentrations of insulin and glucose must be maintained at a certain level in order to satisfy the minimal energy requirements of the body, the concentration of the blood-sugar will not be permitted to decrease until the concentration of the I.F. again rises towards normal levels, that is, the delay in return of the

blood-sugar to its normal value will be inversely proportional to the rate of renewal of the I.F. in the tissues. The prolongation of the blood-sugar curve of a diabetic patient whose capacity to produce insulin is deficient receives therefore a logical explanation.

It might be supposed at first sight that the theory proposed above would demand also an increase in metabolic rate as the result of increasing the concentration of insulin-factor by injection of insulin. The absence of an effect of this kind [Himsworth, unpublished] may be ascribed to the extreme readiness with which the relative excess of glucose is removed from the blood and synthesised into glycogen as the result of the inherent, but varying, capacity of most tissues to effect this object, and not attributed to the existence of a mechanism for the reduction of the effective concentration of the insulin-factor by its storage as an inert substance.

We may regard, then, the rise of blood-sugar in the diabetic as comparable with the rise of blood pressure in chronic interstitial nephritis, and both as compensatory phenomena. The examples need not be multiplied, but the theory offers reasonable explanations of phenomena so far removed as the specific dynamic action of carbohydrates, the results obtained in the combined pancreatectomies and hepatectomies of Mann and Magath, and the constancy of the blood-sugar in the face of diuresis.

The secondary problems arising out of the above primary observations centre around the significance of the linear relationship between the concentrations of sugar in the blood and in the urine. The line itself is an expression of the constant ratio which exists between the height of the blood-sugar above the renal threshold and the concentration of sugar in the urine. There is no similar relationship between the latter and the total blood-sugar.

If we consider the relationship in the light of the secretion theory of renal function, then the renal threshold becomes that level of the blood-sugar required to induce in the tubules the secretion of glucose, and from a consideration of the ratio it would appear that secretion proceeds until an equilibrium is established between the concentration of glucose in the lumen fluid and the concentration of the excess of blood-sugar over the renal threshold.

From the point of view of the filtration-reabsorption theory one may say that the failure of reabsorption is proportional to the excess of blood-sugar over the renal threshold. Ni and Rehberg [1930] have shown that the amount of glucose reabsorbed does not rise until a certain level of blood-sugar is reached and then remain constant, but that reabsorption of glucose goes on even when the blood-sugar is far above the normal. It will be noticed that Ni and Rehberg are speaking in terms of amounts, but if their figures are examined some correspondence will be noticed between the concentrations reabsorbed and the blood-sugar values. It appears quite possible then to interpret their results as indicating that the concentration of sugar reabsorbed by the tubules is governed by the blood-sugar. Above the threshold our own

results support this possibility. We know that the urinary sugar is derived from the blood-sugar and because its concentration is proportional to the excess of the latter above the threshold, it is logical to suppose that any removal of glucose from the filtered fluid in its passage through the tubules occurs also in a concentration proportional to the head of blood-sugar above the threshold. The ratio of excess of blood-sugar to urinary sugar concentration is constant above the threshold, and, if the above argument is correct, then the ratio of excess of blood-sugar over the threshold to concentration of reabsorbed fluid is also constant, and as sugar is escaping and appearing in the urine this can only mean that above the threshold the maximum "reabsorption ratio" has been brought into play. Now the excess of sugar is but a fraction of the whole blood-sugar, and it is unreasonable to imagine that the effect of this excess upon the lumen fluid is different from that exercised by the remainder. Thus it would appear that below the threshold the blood-sugar still governs the concentration of the glucose reabsorbed from the glomerular filtrate. The expression $\frac{B-x}{W} = K$ is mathematically untenable in this latter situation and certainly, if the tubules are working at the maximum reabsorption ratio, they are absorbing sugar at a greater concentration than they need to avoid glycosuria. Theoretically this would leave an excess of water in the tubules and if one considers that many volumes of blood must be filtered to provide one volume of urine then the excess would be considerable, and one would expect a diuresis occurring before the threshold was passed to diminish progressively as sugar appeared. But, in experiments in which the intake of water per unit of time is kept constant, this is the reverse of experience. Apparently then the maximum reabsorption ratio does not come into play until the renal threshold is reached, and in that case the renal threshold may be defined as that value of the blood-sugar required to induce the maximum ratio of reabsorption between the concentration of the blood-sugar and the concentration of sugar in the reabsorbed fluid.

The greater the change in concentration of urinary sugar per unit change in concentration of the blood-sugar the more nearly horizontal the line in the graph, and the smaller the relative change the more nearly vertical the line. The inclination of the line or the value of the ratio might thus be regarded as an indication of the permeability of the kidney to glucose. This does not appear to be characteristic of any group of cases and on the basis of it no differentiation of glycosuria into types can be made. The ratio, however, is constant either on the rise or on the fall in any particular experiment, and as it determines the renal threshold, it establishes conclusively the existence of two separate thresholds, a threshold of appearance and a threshold of disappearance. On the mechanism of their differentiation, however, it throws no light.

Reference must now be made to an interesting discrepancy which occurred during the course of the work.

An investigation was carried out in the usual manner, save that no water was given to the patient after the administration of the glucose solution. The

results are charted in Figs. 10 and 11. The blood- and urinary-sugar curves taken on the rise do not run parallel (Fig. 10) and it will be seen that sugar was

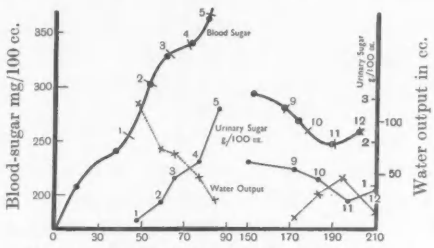


Fig. 10.

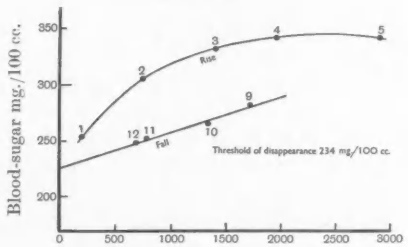


Fig. 11. Linear graph corresponding to Fig. 10.

excreted in concentrations which increased in unexpected proportion to the blood-sugar. After the first specimen the diuresis fell off markedly. In Fig. 11 are shown the lines obtained on charting the two sets of values. The figures obtained on the rise fall on a curve whilst those obtained on the fall lie on the usual straight line. In all the experiments previous to this, determinations had been made on the fall, no attention paid to diuresis, and perfect results obtained. Also in figures taken from the literature with slowly rising blood-sugar curves no discrepancy had been observed. It appears then that with a rapidly rising blood-sugar curve, to obtain correspondence with the urinary sugar, a diuresis is essential.

The interpretation of this observation appears to be as follows. Fluid is filtered off at the glomerulus at a definite level of the blood-sugar, and if owing to diuresis it passes rapidly along the lumen, then when it reaches the tubules it is subjected to the influence of blood of approximately the same concentration as its own precursor and is concentrated proportionately. If, however, it passes slowly down the tubules, whilst the sugar content of the blood is rising rapidly, then it is exposed in the tubules to a blood-sugar value much higher than that at which it was filtered and is concentrated excessively. The result will be a failure of proportionality between urine and blood-sugar figures, and with a progressive fall in diuresis there will be a progressive rise

in the disproportion. On the fall the same argument would explain the occurrence of a more dilute urine than was expected in association with a given blood-sugar, but the slower decrease of the blood-sugar on the fall does not predispose to widely varying sugar contents in the glomerular and peritubule bloods, and thus the discrepancy will tend not to occur.

Poulsson [1930] has shown that if the tubules are paralysed by phlorhidzin sugar behaves as a non-threshold substance, that is, it is excreted *in toto* and the whole mechanism of proportionate excretion previously described presumably breaks down. Thus the tubules appear to be the seat of the proportionate concentration mechanism and in the light of the above experiment it appears justifiable to conclude that the controlling factor of this mechanism is the sugar content of the blood in the peritubule capillaries.

Summary.

1. A review of the literature and methods previously used in the determination of the renal threshold for glucose is given.

2. A new method is described for determining the renal threshold accurately.

3. The results of calculations made from figures taken from the literature and from those obtained in the present investigation are recorded.

4. It is demonstrated that the concentration of urinary sugar is independent of the diuresis, and that it is proportional to the excess of the blood-sugar concentration over the renal threshold.

5. The nature of the renal threshold is discussed in the light of these results and a theory is advanced to explain the lack of dependence of the urinary sugar concentration on the diuresis.

6. Evidence is produced that the concentration of the sugar reabsorbed is determined by the sugar content of the blood in the peritubule capillaries.

In conclusion I should like to thank Sister Freeman for the great assistance she has given me in obtaining urine specimens from female patients, to express my appreciation to Prof. T. R. Elliott for permission to investigate his patients, and to record my gratitude to Prof. C. R. Harington for his help and criticism.

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CXXIX. STUDIES ON THE RELATIONSHIP BETWEEN CHEMICAL CONSTITUTION AND PHYSIOLOGICAL ACTION.

III. THE INHIBITORY ACTION OF CERTAIN SYNTHETIC URETHANES ON THE ACTIVITY OF LIVER ESTERASE.

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Parts I and II [Stedman, 1926; 1929, 1] of this series of communications have dealt with a new relationship between chemical constitution and physiological action. It has been shown that certain synthetic urethanes possess in common with the alkaloid physostigmine, which is also a urethane, the power of producing a constriction of the pupil when instilled into the eye. As a result of this work, as well as of extensions thereof published elsewhere [Stedman and Stedman, 1929; 1931], it has been concluded that substances which can be classed as phenyl esters of carbamic acids and which possess a basic group will, in general, exhibit similar miotic properties. Constitutional factors which repress, or tend to repress, such properties have already been discussed. Until recently the pharmacological examination of the synthetic urethanes had not extended beyond their action on the eye and it was not therefore certain that they would resemble physostigmine in other physiological properties. One of the urethanes, namely, the methylurethane of *α-m*-hydroxyphenylethyldimethylamine, which had already been shown to possess marked miotic properties and had been named miotine [Stedman, 1929, 2] on that account, has, however, now been submitted to a complete, and three others to an extensive although less complete, pharmacological examination [White and Stedman, 1931]. Miotine has thus been shown to possess physiological properties which are qualitatively identical with those of physostigmine, and similar results have been obtained with the other urethanes as far as they have been examined. The above relationship between chemical constitution and physiological action is therefore not limited to miotic activity but embraces all the known physiological actions of physostigmine. Now, Loewi and Navratil [1926] have attributed the action of physostigmine on the heart to its power of inhibiting the destruction of acetylcholine by an agent which they have shown to be present in the heart and in aqueous extracts of this organ. A similar agent is present in the blood of certain species [Galehr and

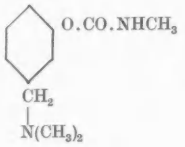
Plattner, 1927]. Loewi and Navratil consider this agent to be an esterase, a view which has been disputed by Plattner and Galehr [1928]. The arguments of Plattner have, however, been disposed of in a recent paper by Engelhart and Loewi [1930], who have brought forward convincing evidence of the enzymic nature of the substance causing the destruction of acetylcholine. Evidence leading to the same conclusion has also been published by Matthes [1930]. In view of the similarity between the actions of miotine and physostigmine on the heart, and in particular of the fact that miotine, like physostigmine, sensitises the vagus and potentiates the action of acetylcholine, it might be expected that miotine would also inhibit the destruction of acetylcholine by the agent in question. That this is actually the case has been shown by Dr Matthes, who kindly compared the action of miotine with that of physostigmine during his investigation of the inhibition by the latter of the destruction of acetylcholine by serum. The problem thus arises as to whether urethanes of the type discussed above, which have been shown to possess similar physiological properties, also share the property of inhibiting the action of the serum-enzyme. Another problem is, however, involved. In the investigations both of Loewi and his co-workers and of Matthes, the destruction of acetylcholine by the solutions under examination has been measured by biological methods, and no experiments appear to be recorded which indicate whether the enzyme which is responsible for this destruction will hydrolyse simple esters, or whether its action is specific towards acetylcholine. If the enzyme is a true esterase it should be capable of hydrolysing simple esters, in which case its activity, as well as the inhibition thereof by drugs, could be followed by titration methods. It appeared to us that these problems could be most conveniently, although indirectly, solved by examining the influence of the urethanes on the activity of the enzyme present in the liver and which is known to hydrolyse simple esters more readily than glycerides. The results of such an investigation are recorded in the present communication.

Materials.

Esterase preparations. All the esterase solutions employed in these experiments have been prepared from pigs' livers by the method of Willstätter and Memmen [1924, 2]. The liver, obtained direct from the slaughter house, was finely minced, desiccated by successive treatment with acetone and ether, and the dry preparation powdered and sieved. Solutions of the enzyme were prepared, as required, by extracting 2 g. of this fine powder with 100 cc. of 0·025 *N* ammonia for 1 hour and removing the insoluble material in the centrifuge. In the earlier experiments no further purification of the esterase was attempted. In the later work, however, it was found to be advantageous carefully to acidify the ammoniacal extract with acetic acid, and, after removing the precipitate so produced, to dialyse the clear solution in collodion membranes for 3–4 days, a procedure similar to that first described by

Willstätter, Bamann and Waldschmidt-Graser [1928] and subsequently employed by other workers.

Substances examined for inhibitory action. The urethanes with a pharmacological action similar to that of physostigmine which have been examined for inhibitory action comprise three series of isomeric compounds, namely, the methylurethanes of *o*-, *m*- and *p*-hydroxybenzyldimethylamine, the *o*-, *m*- and *p*-dimethylaminophenyl esters of methylcarbamic acid, and the methylurethanes of the isomeric *α*-hydroxyphenylethyldimethylamines. The structures of the *m*-isomerides in these three series are represented in formulae I, II and III respectively. These compounds have been examined both in the



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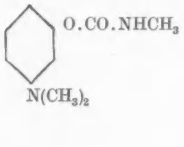

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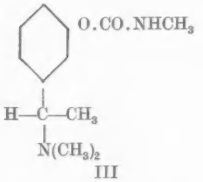

III

form of their hydrochlorides and methiodides, the preparation of which has been effected by the methods of Stedman [1926; 1929, 1] and Stedman and Stedman [1929]. In the case of the *o*-isomerides, the hydrochlorides of which are hygroscopic, the base was dissolved in the calculated quantity of hydrochloric acid immediately before use. It should be noted that the urethane represented by formula III is the substance which has been named miotine. As will be evident from the formula, miotine and its position isomerides contain an asymmetric carbon atom. Unfortunately attempts to resolve these compounds have not so far met with success; the examination of their inhibitory actions towards liver esterase has therefore necessarily been carried out with the racemic substances. It will be convenient in the following pages to refer to the isomeric compounds corresponding with formula III as *o*-, *m*- and *p*-miotine, and to those corresponding with formulae I and II as the methylurethanes of the benzyl and phenyl series respectively.

In addition to urethanes with a physostigmine-like action, the methylurethane of choline iodide, $CH_3NH.CO.OC_2H_4N(CH_3)_3I$ [Stedman, 1929, 1], has been examined as an example of an aliphatic urethane. Some tests have also been made with the following alkaloids: pilocarpine, arecoline, ricinine. The two former were employed as nitrate and hydrobromide respectively, these salts being obtained from commercial sources. The ricinine was prepared from castor-oil seeds; since this alkaloid is soluble in water it was employed in the form of the free base.

Pilocarpine and arecoline were examined because the pharmacological actions of these alkaloids are similar to that of physostigmine. Ricinine does not exhibit such similarities. It occurs, however, in the castor-oil seed associated with a lipase, and since physostigmine also occurs in a seed, the Calabar

bean, it was thought that if the two alkaloids were found to resemble one another with respect to their inhibition of the activities of enzymes, this might form the basis of an explanation of the functions of alkaloids in general. As will be shown below, ricinine does not inhibit the activity of liver esterase; its possible influence on the activity of ricinus lipase, the enzyme with which it is associated naturally, has not yet been investigated.

Experiments with methyl butyrate as substrate.

Technique. The activity of the esterase preparations has been determined by a method essentially similar to that developed by Willstätter and Memmen [1924, 1] for pancreatic lipase and subsequently adapted by the same authors [1924, 2] for use with liver esterase. For convenience of measurement, 1 cc. of substrate (methyl butyrate) was used for each 100 cc. of reaction mixture, as recommended by Bamann [1929]. The general procedure was as follows: 20 cc. of ammonia-ammonium chloride buffer (1 part 2·5 *N* NH_3 : 2 parts 2·5 *N* NH_4Cl; p_H 8·9) contained in a 100 cc. measuring flask were diluted with 65–70 cc. of water, the exact volume depending on the volume of enzyme solution subsequently added, and the mixture brought to 30° in a thermostat. 1 cc. of methyl butyrate was then added and the flask shaken vigorously until the ester had dissolved completely. To the clear solution a measured volume (5 or 10 cc.) of the enzyme preparation was added, the total volume of the mixture brought to 100 cc. by the addition of water, and the whole thoroughly mixed. 20 cc. of the mixture were immediately withdrawn, run into a mixture of 25 cc. of 0·2 *N* hydrochloric acid with 20 cc. of water, and titrated with approximately 0·2 *N* sodium hydroxide, using bromocresol purple as indicator. Similar titrations were made at intervals of 20 minutes. With this procedure and using a suitable quantity of enzyme the amount of hydrolysis was approximately proportional to the time.

The enzyme solution consisted, in the earlier experiments, when ammoniacal extracts of liver powder were employed, of 5 cc. of a mixture of 5 cc. of the extract with 1 cc. of water, the latter being replaced by 1 cc. of a solution of the substance under examination in the experiments designed to test the inhibitory action of this substance. In the later experiments, in which the acidified and dialysed esterase preparation was employed, 5 cc. of enzyme solution were mixed with 5 cc. of ammonia-ammonium chloride buffer (of above composition but diluted 1 in 10), 2 cc. of water or solution added, and 10 cc. of this mixture used for the hydrolysis.

Activity of esterase preparations. The activity of the esterase preparations made from different livers was fairly constant. The following figures, which represent the number of cc. of 0·194 *N* alkali required to titrate the acid liberated in 20 cc. of the reaction mixture in 20, 40 and 60 minutes respectively, using 4·17 cc. of an ammoniacal extract of liver powder in 100 cc. of reaction mixture, may be taken as typical: 2·3, 4·75, 7·25. This result was

obtained on May 19th, 1930, with a fresh extract of a liver powder (L.P. 2) prepared on April 23rd, 1930, and almost identical values were obtained on June 10th, 1930, with a similar extract from the same powder. These facts are recorded in detail because of the following remarkable phenomenon which was observed with an extract of this powder but which has not so far been encountered with other preparations. On June 23rd, 1930, a fresh ammoniacal extract of L.P. 2 was rendered just acid to litmus by the addition of 0·5 *N* acetic acid. The precipitate, which formed slowly, was centrifuged off and the clear solution tested for activity under precisely the same conditions as those employed for the above ammoniacal extract, when the following figures were obtained: 4·45, 8·25, 8·95. Owing to the unexpectedly high activity of this extract the hydrolysis of the substrate was virtually complete in the experimental period of 1 hour, with the result that the reaction did not take the usual linear course. It is therefore not possible accurately to compare the activities of the different extracts of L.P. 2, but it is clear from the figures quoted that the activity of the acidified extract was at least twice as great as that of the ammoniacal extracts previously prepared from the same weight of the same powder. Dialysis of the acidified extract for 4 days was accompanied by an apparent slight diminution in activity, as is shown by the following titration figures: 3·4, 6·7, 8·35; this, however, was mainly due to the dilution which occurred during dialysis. The increase in activity observed on acidification can doubtless be explained by the removal of an inhibitory substance; from the difficulty in reproducing this experiment it appears that a very critical adjustment of conditions, probably of p_H, is necessary for the precipitation of this inhibitor.

Influence of time of contact between inhibitor and enzyme on inhibitory action. Rona and Bach [1920], in their studies on the inhibitory action of atoxyl on various lipases, have shown that the maximum inhibitory effect of this substance is not developed unless it is left for a certain period of time in contact with the enzyme before the addition of the substrate. A number of preliminary experiments having demonstrated that urethanes of the miotine type exert a pronounced inhibitory action on the hydrolysis of methyl butyrate by liver esterase, the influence of this time of contact of the inhibitor with the enzyme was investigated. For this purpose, 1 cc. of a solution of miotine hydrochloride, containing 1·2 mg./cc., was added to each of 7 test-tubes containing 5 cc. of an ammoniacal extract of liver powder. After varying intervals of time 5 cc. of the mixture were employed for a hydrolysis experiment. The results are given in Table I. The figures indicate that, under the conditions employed, the maximum inhibitory action of miotine hydrochloride is not exerted unless it has been in contact with the esterase for 2 hours prior to the addition of the substrate. The difference between the maximum inhibitory action and that produced after contact for 1 hour is, however, very small and almost falls within the limits of experimental error. It has therefore been considered sufficient, when comparing the inhibitory activities of the various

Table I. *Influence on inhibitory action of time of contact of inhibitor with enzyme.*

Substrate: methyl butyrate. Inhibitor: miotine hydrochloride. T = 30°.

Time of contact (mins.)	Titration figures (cc. of 0·194 *N* NaOH required to titrate acid liberated in 20, 40 and 60 mins.)			Percentage inhibition
Control (no inhibitor)	2·5	5·15	7·75	—
0·1	1·8	3·7	5·6	28
15	0·9	1·95	3·0	61
30	0·75	1·5	2·25	71
45	0·6	1·25	1·9	75
60	0·5	1·1	1·7	78
120	0·4	0·9	1·5	81
330	0·4	0·9	—	81

urethanes, to allow the inhibitor to stand in contact with the enzyme for 1 hour before use.

The figures in Table I give some further important information. Miotine is an ester of methylcarbamic acid and might therefore be expected to suffer, like other esters, a more or less rapid hydrolysis when in contact with liver esterase. In the above experiments, however, it is clear that no appreciable hydrolysis has occurred in a period of 5½ hours. In this connection it should be mentioned that, although the hydrolysis experiments were carried out at 30°, the mixtures of miotine and esterase were allowed to stand at room temperature before addition to the reaction flask.

Some experiments on the influence of the time of contact of enzyme and inhibitor on the inhibitory action of the latter were also made under somewhat different conditions. Although the procedure employed in these experiments was not adopted in much of the subsequent work, the results are recorded here since they are not entirely without interest. In the preceding experiments the enzyme and inhibitor were in contact under conditions of concentration widely different from those obtaining in the final reaction mixture. In order to render these conditions more nearly comparable the following modified series of experiments was carried out. A solution of 1·2 mg. of miotine hydrochloride in 1 cc. of water was added to each of a series of flasks containing the diluted buffer mixture and enzyme (ammoniacal extract) previously warmed to 30°. After varying intervals of time, 1 cc. of methyl butyrate was added and rapidly dissolved by shaking, the volume adjusted to 100 cc., and the titrations were performed as before. The results are expressed in Table II. Unfortunately a quantitative comparison cannot be made between the results of Tables I and II since not only were different enzyme preparations employed but the amounts of inhibitor used were slightly different. Nevertheless it is evident that the inhibitory action of miotine hydrochloride is of the same order of magnitude in the two sets of experiments. The most striking point of difference is the definite indication of a destruction of the miotine in the second set after 4 hours' contact with the enzyme. We are inclined, however, to attribute this destruction to the alkalinity of the

Table II. *Influence on inhibitory action of time of contact of inhibitor with enzyme.*

Substrate: methyl butyrate. Inhibitor: miotine hydrochloride. T = 30°.

Time of contact (mins.)	Titration figures (cc. of 0·194 *N* NaOH required to titrate acid liberated in 20, 40 and 60 mins.)			Percentage inhibition
Control (no inhibitor)	1·95	4·00	6·1	—
15	0·7	1·5	2·35	61
30	0·6	1·25	1·7	72
45	0·4	1·0	1·4	77
60	0·4	0·85	1·3	79
120	0·45	1·0	1·6	74
240	0·8	1·65	2·45	60

solution, combined with the fact that the enzyme and inhibitor were maintained at 30° during the whole period of contact, rather than to the action of the esterase.

Inhibitory actions of various urethanes. A number of the urethanes exhibiting miotic activity were examined for inhibitory action under fairly wide ranges of concentration of inhibitor. The results are collected in Table III.

Table III. *Inhibitory action of various urethanes with miotic activity.*

Substrate: methyl butyrate. T = 30°. Initial $p_H = 8·9$.

In the three instances marked (*a*), 10 cc. of the extract were employed owing to the diminution in the activity of the liver powder.

The solution used in the experiment marked (*b*) was prepared by extracting only 1·5 g. of liver powder with 100 cc. of *N*/40 ammonia.

Inhibitor	Liver preparation	Final conc. of inhibitor (mg. per 100 cc.)	Titration figures			Percentage inhibition
m-Miotine HCl	L.P. 2	Control	2·55	5·15	7·8	—
		10	0·2	0·5	0·9	89
		1	0·45	1·0	1·45	81
		0·1	1·25	2·6	4·05	48
		0·01	2·1	4·3	6·55	16
		0·001	2·4	4·8	7·3	6
o-Miotine HCl	L.P. 5 (*a*)	Control	2·15	4·35	6·5	—
		10	0·55	1·0	1·45	78
		1	1·6	3·3	4·9	25
		0·1	1·8	3·75	5·9	11
p-Miotine HCl	L.P. 2	Control	2·35	4·75	7·1	—
		10	0·0	0·3	0·45	94
		1	0·25	0·55	0·85	88
		0·1	0·9	1·5	2·55	64
		0·01	1·65	3·3	5·0	30
		0·001	2·35	4·5	6·85	4
m-Miotine MeI	L.P. 2	Control	2·45	5·0	7·5	—
		10	0·25	0·5	0·85	90
		1	1·25	2·4	3·75	51
		0·1	2·15	4·4	6·75	11
		0·01	2·15	4·8	7·2	4
		0·001	2·3	4·8	7·3	—
o-Miotine MeI	L.P. 5 (*a*)	Control	2·0	4·2	6·4	—
		10	0·25	0·7	1·1	83
		1	1·2	2·5	3·6	44
		0·1	2·05	4·1	6·2	3

Table III (*contd.*).

Inhibitor	Liver preparation	Final conc. of inhibitor (mg. per 100 cc.)	Titration figures			Percentage inhibition
p-Miotine MeI	L.P. 5 (*b*)	Control	1·75	3·4	5·2	—
		10	0·15	0·35	0·65	86
		1	0·85	1·55	2·35	55
		0·1	1·4	3·0	4·5	14
		0·01	1·6	3·25	5·0	—
		0·001	1·45	3·3	5·0	—
HCl of *o*-urethane (phenyl series)	L.P. 6	Control	2·5	5·1	7·7	—
		10	0·2	0·6	1·0	87
		1	0·6	1·2	1·85	77
		0·1	1·5	3·05	4·75	38
		0·01	2·05	4·15	6·4	17
		0·001	2·2	4·55	7·05	8
HCl of *m*-urethane (phenyl series)	L.P. 2	Control	2·45	4·85	7·5	—
		10	0·0	0·1	0·3	96
		1	0·35	0·5	0·75	90
		0·1	1·15	2·1	3·25	57
		0·01	1·8	3·55	5·45	27
		0·001	2·1	4·6	6·8	9
HCl of *p*-urethane (phenyl series)	L.P. 2	Control	2·3	4·75	7·4	—
		10	0·25	0·5	0·85	89
		1	0·7	1·35	2·0	73
		0·1	1·7	2·25	3·95	33
		0·01	2·2	4·4	6·65	11
		0·001	2·2	4·55	7·0	6
MeI of *m*-urethane (phenyl series)	L.P. 2	Control	2·45	5·1	7·6	—
		10	0·75	1·5	2·4	69
		1	1·7	2·35	3·95	37
		0·1	2·15	4·55	6·9	11
		0·01	2·25	4·75	7·2	5
		0 001	2·35	4·8	7·3	—
MeI of *p*-urethane (phenyl series)	L.P. 5 (*a*)	Control	2·1	4·4	6·55	—
		10	0·6	1·15	1·7	74
		1	1·35	2·65	4·0	39
		0·1	2·2	4·3	6·45	—
MeI of *o*-urethane (benzyl series)	L.P. 2	Control	2·35	4·75	7·3	—
		10	0·2	0·45	0·75	90
		1	1·15	2·3	3·4	53
		0·1	2·3	4·6	7·0	4
		0·01	2·4	4·75	7·25	—

In all these experiments the general technique described earlier was utilised, ammoniacal extracts of desiccated liver powder being employed as esterase solution in each case. Since this work was carried out over an extended period of time, different liver preparations, as indicated in Table III, were necessarily employed owing to the moderately rapid deterioration which such preparations undergo. The results demonstrate clearly the marked inhibitory action which urethanes of the type examined exert upon the activity of liver esterase. A detailed comparison of the figures shows further that, in general, the inhibitory activity of the hydrochlorides of the tertiary bases is greater than that of the corresponding quaternary iodides. While this is not universally the case, particularly in the higher concentrations, the inhibitory activities of the methiodides consistently decrease, with increasing dilution of the inhibitor, at a much greater rate than do those of the hydrochlorides of the tertiary

bases, with the result that at low concentrations the latter are uniformly more active. This difference between the two types of salts is emphasised because it is probably related to the mechanism of the inhibitory action.

Examination of other types of compounds for inhibitory activity. The following compounds were tested for inhibitory activity under conditions similar to those used in the preceding experiments: methylurethane of choline iodide, hydrochloride of *α-m*-hydroxyphenylethyldimethylamine, pilocarpine nitrate, arecoline hydrobromide, atropine hydrochloride and ricinine. The titration figures need not be recorded in detail since in no case was any inhibitory action observed in the highest concentrations examined, namely, 10 mg. per 100 cc. of reaction mixture.

Influence of p_H *on inhibitory action.* The technique adopted for the experiments in which methyl butyrate was employed as substrate is not very suitable for the investigation of p_H effects. Even when relatively large amounts of buffer are employed, the acid liberated during the hydrolysis is sufficient to cause some change in p_H; if the solution is thereby brought to the acid side of neutrality, some loss in the activity of the esterase may occur with the result that the hydrolysis of the substrate will no longer proceed at a constant rate. This effect was actually observed in a number of experiments. In the experiment quoted in Table IV, which was carried out at p_H 6·8, proportionality between the amount of hydrolysis and the time was maintained;

Table IV. *Influence of* p_H *on the inhibitory action of miotine hydrochloride.*

Substrate: methyl butyrate. T=30°.

p_H	Esterase preparation	Conc. of inhibitor (mg. per 100 cc.)	Titration figures			Percentage inhibition
6·8	Acidified ammoniacal	Control	1·65	3·4	5·0	—
(phosphate buffer)	extract of L.P. 5	10	0·5	1·25	2·0	60
		1	1·45	2·9	4·35	13
		0·1	1·6	3·2	4·85	3
		0·01	1·75	3·4	4·95	—
		0·001	1·7	3·5	5·15	—
8·9	Ammoniacal extract	Control	1·85	3·8	5·75	—
(ammonia buffer)	of L.P. 5	12	0·05	0·25	0·45	92
		1·2	0·35	0·75	1·15	80
		0·12	1·0	2·25	3·5	39
		0·012	1·65	3·3	4·9	15
		0·0012	1·85	3·6	5·45	5

this experiment therefore serves to illustrate the general influence of p_H on the inhibitory action of miotine hydrochloride. For comparison, the results of an experiment at p_H 8·9 carried out under similar conditions are included in the same table. The technique described in connection with the experiments quoted in Table II was employed for both experiments. The phosphate buffer used consisted of a mixture of equal parts of $M/3$ Na_2HPO_4 and $M/3$ NaH_2PO_4, 70 cc. being employed in each 100 cc. of reaction mixture. When this buffer was employed, phenolphthalein was used as indicator for the titrations.

Unfortunately the enzyme solutions employed in these experiments, although prepared from the same liver powder, were of different degrees of

purity; the results are not therefore strictly comparable. Nevertheless, two important conclusions can be drawn from these experiments: firstly, the inhibitory action of miotine hydrochloride is smaller on the acid than on the alkaline side of neutrality; secondly, the inhibitory action falls off with decreasing concentration of inhibitor much more rapidly in acid than in alkaline solution.

Comparison of the inhibitory activities of isomeric urethanes. The urethanes which have been shown above to inhibit the hydrolysis of methyl butyrate by liver esterase are members of three series of isomeric compounds, with their corresponding methiodides. The results do not, however, necessarily indicate the relative inhibitory activities of the position isomerides within a given series or even of members of different series, for, as previously mentioned, the inhibitory activities of the various compounds were examined on different esterase preparations, and since the latter were made from different liver powders at varying times after their preparation they possessed different activities and probably contained varying amounts of natural inhibitors. On the assumption that any natural inhibitors which may be present in the enzyme preparation enter into competition with the urethanes, it is clear that in experiments designed to compare the activities of different compounds it is not sufficient to employ the same esterase activity; the comparison can only be regarded as valid in those cases where the same esterase extract has been employed. Some experiments, recorded in Table V, have consequently been carried out in which a comparison has been made of the inhibitory activities of the members of each series of urethanes towards the same enzyme preparation. Finally, the hydrochlorides of the *m*-isomerides of the three series have been compared under the same conditions as a measure of the relative activities of the three series of compounds. In all cases the ammoniacal extracts of the liver powders were purified by acidification with acetic acid followed by dialysis.

From the magnitude of the titration figures it is evident that the percentage inhibition shown in the last column of Table V must be subject to an error of a few per cent. Bearing this in mind, the more significant results revealed by a study of this Table may be summarised as follows.

Miotine series. The inhibitory activities of the hydrochlorides of the *m*- and *p*-isomerides are identical, that of the *o*-isomeride being considerably smaller; the inhibition produced by the latter is, in fact, slightly smaller than that caused by the two former when present in one-tenth of its concentration. No very great difference can be observed between the activities of the methiodides, the *m*- and *p*-isomerides showing about equal activity and the *o*-compound exhibiting a slightly greater effect. It is noteworthy and exceptional that the inhibitory activity of the hydrochloride of the *o*-isomeride is somewhat smaller than that of its methiodide in the same molar concentration.

Phenyl series. The activities of the hydrochlorides are definitely in the order $m > p > o$. The methiodides follow the same order; the *m*- and *p*-compounds show, however, practically identical activities.

Table V. *Comparison of inhibitory activities of various urethanes.*

Substrate: methyl butyrate. T = 30°. Initial p_H = 8·9.

Inhibitor (methylurethane)	Esterase preparation	Final conc. of inhibitor (molar × 10^{-3})	Titration figures			Percentage inhibition
Miotine series:	L.P. 6	Control	1·75	3·5	5·3	—
m-HCl		1/30	0·25	0·65	1·95	80
,,		1/300	1·15	2·4	3·7	30
p-HCl		1/30	0·4	0·8	1·2	77
,,		1/300	1·25	2·45	3·85	27
o-HCl		1/3	0·75	1·55	2·3	57
,,		1/30	1·5	2·95	4·5	15
m-MeI		1/3	0·7	1·35	1·9	63
,,		1/30	1·65	3·2	4·9	8
p-MeI		1/3	0·75	1·15	1·85	65
,,		1/30	1·4	2·9	4·4	17
o-MeI		1/3	0·4	0·8	1·25	77
,,		1/30	1·45	2·9	4·3	19
Phenyl series:	L.P. 6	Control	1·6	3·35	5·1	—
m-HCl		1/300	0·15	0·45	0·7	86
p-HCl		,,	0·8	1·65	2·6	49
o-HCl		,,	1·0	2·05	3·2	37
m-MeI		1/30	0·9	1·75	2·55	52
p-MeI		,,	0·75	1·6	2·55	50
o-MeI		,,	1·3	2·6	4·0	22
Benzyl series:	L.P. 7	Control	2·4	4·75	6·85	—
m-HCl		1/300	0·8	1·75	2·55	63
p-HCl		,,	1·35	2·75	3·95	42
o-HCl		,,	1·85	3·6	5·7	17
m-MeI		1/30	1·45	2·8	4·3	37
p-MeI		,,	0·75	1·5	2·25	67
o-MeI		,,	1·25	2·55	3·85	44
All series:	L.P. 7	Control	2·15	4·55	6·75	—
m-Benzyl-HCl		1/3000	1·95	3·8	5·85	13
m-Phenyl-HCl		,,	1·15	2·45	3·6	47
m-Miotine-HCl		,,	1·5	3·1	4·7	30
	L.P. 7	Control	2·4	4·75	6·85	—
m-Benzyl-HCl		1/300	0·8	1·75	2·55	63
m-Phenyl-HCl		,,	0·1	0·35	0·45	93
m-Miotine-HCl		,,	0·35	0·6	0·8	88

Benzyl series. In this series the order for the hydrochlorides is $m > p > o$, and for the methiodides $p > o > m$.

Comparison of the three series. The inhibitory activities of the hydrochlorides of the *m*-isomerides in equimolar concentrations are definitely different, the order being phenyl > miotine > benzyl.

The results in Table V may be further utilised in order to direct attention to the varying activities of the urethanes with different esterase preparations. Thus, to take the most marked case, the concentrations of miotine hydrochloride required to inhibit the acidified and dialysed extracts of two esterase preparations (L.P. 6 and L.P. 7) to exactly the same extent (30 %) are in the ratio 1:10, the extract from L.P. 6 requiring a final concentration of 1/300,000 *M* and that from L.P. 7 one of 1/3,000,000 *M*. The different responses of these enzyme preparations are doubtless related to their histories, which are as follows: the liver powder L.P. 6 was prepared on January 9th,

1931. An ammoniacal extract of this was made on January 14th and its activity tested by the standard procedure used in this investigation, when the following titration figures were obtained: 2·3, 4·6, 7·0. Practically identical values were obtained on the same day following the acetic acid treatment. The acid solution was then dialysed for 5 days, the dialysed solution giving, on January 19th, titration figures of 2·05, 4·05, 6·05. The diminution in activity shown by these figures is only apparent and can be accounted for by the slight dilution which the extract had undergone during dialysis. On the following day, however, the titration figures for the dialysed extract had fallen to 1·75, 3·5, 5·3. Thus, in 24 hours the activity had diminished about 12 %, and it was this extract with lowered activity which was employed for the comparison of the inhibitory activities of the urethanes of the miotine series. We are of opinion that this loss in activity can be attributed to the acidity caused by the relatively long period of dialysis, and we have found that, with the highly permeable collodion bags which we employ, such losses consistently occur when the process of dialysis is extended beyond 3 or, at most, 4 days.

The preparation L.P. 7 was made on February 18th, 1931. An ammoniacal extract was prepared on February 20th, and this was acidified and dialysed until February 23rd. In view of the fact that this extract, which was more sensitive to miotine hydrochloride than the one referred to above, proved to be stable, it appears probable that the presence of inactivated esterase in the enzyme solutions diminishes the inhibitory activity of urethanes of the type under discussion.

Experiments with tributyrin as substrate.

Technique. In order to follow the hydrolysis of tributyrin by liver esterase the stalagmometric method devised by Rona and Michaelis [1911] and subsequently employed by Willstätter and Memmen [1923] in connection with pancreatic lipase has been utilised. A straight stalagmometer with a water value of 81 at 20° was employed throughout the experiments.

In using this method it is frequently considered sufficient to work at room temperature without any special temperature control. At the time our experiments were carried out, however, the temperature of our laboratory fluctuated considerably and tended to be somewhat low, falling at times to below 10°. At such temperatures the activity of the enzyme is naturally considerably diminished, and it was therefore considered advisable to use a thermostat. The reaction mixture, contained in a small beaker, was placed in a bath at 20° and the stalagmometer supported directly over the beaker by means of a suitable stand. By raising the beaker and applying gentle suction at the top of the stalagmometer the mixture could be drawn into the latter. The beaker was then again lowered and the drops from the stalagmometer allowed to fall into it. By this arrangement fluctuations in the temperature of the liquid actually in the stalagmometer could not be prevented, but the

bulk of the reaction mixture was maintained constantly at 20°, thereby permitting a uniform action of the esterase.

The remaining procedure was as follows: 50 cc. of a saturated solution of tributyrin were mixed with 5 cc. of $M/3$ phosphate buffer (p_H 7·9 or 6·8) and the mixture brought to 20°. 1 cc. of a mixture of 1 cc. of esterase solution with 1 cc. of water in the control experiment, or of 1 cc. of esterase solution with 1 cc. of a solution of the urethane in the experiments designed to measure the inhibitory action of the latter, was then added and the drop number immediately measured. Two further counts were made at intervals of 20 minutes. As in the experiments with methyl butyrate, the enzyme was left in contact with the urethane for 1 hour before mixture with the substrate.

All the esterase solutions employed were prepared from ammoniacal extracts of liver powders acidified with acetic acid; in some cases the extracts were dialysed. In each case, 1 to 5 cc. of the enzyme preparation, the actual volume depending on its activity, were mixed with 2 cc. of $M/3$ phosphate buffer of the same p_H as that employed in the hydrolysis experiments, and diluted to 10 cc. with water.

Willstätter and Memmen [1923] have pointed out that commercial preparations of tributyrin frequently contain an impurity, with a saponification number corresponding with that of dibutyrin, which renders them unsuitable for use, without extensive purification, in the stalagmometric estimation of lipolytic activity. As a criterion of purity they recommend the preparation of two saturated aqueous solutions of the sample, one by shaking 5 to 10 g. of the tributyrin with 200 cc. of water, and the other by shaking 3 drops with a similar volume of solvent. If the tributyrin is pure, the two solutions should give the same drop number. The experiments recorded in this communication have been made with one delivery of B.D.H. tributyrin. When this was submitted to the test described above, the two saturated aqueous solutions gave identical drop numbers. According to the above criterion the sample was therefore quite pure and was employed in our experiments without further treatment.

Influence of p_H *on inhibitory action.* The study of the inhibition of the hydrolysis of tributyrin by liver esterase by urethanes of the type considered in this series of communications has been carried out partly with the object of ascertaining if the inhibitory action of such urethanes is independent of the type of substrate employed and partly because, owing to the sensitivity of the stalagmometric method, only relatively small amounts of tributyrin need be employed as substrate. This renders the method eminently suitable for a study of the influence of p_H on the inhibitory process, since no difficulty is experienced in buffering the small amounts of acid which are liberated by the action of the enzyme. The inhibitory activities of the hydrochlorides and methiodides of each member of the three series of urethanes mentioned above have therefore been examined, using a range of concentrations of inhibitor, at two different acidities, namely, p_H 7·9 and 6·8. Table VI gives some typical

Table VI. *Influence of* p_H *on the inhibitory action of various urethanes.*

Substrate: tributyrin. T = 20°.

Inhibitor	Final conc. of inhibitor (mg. per 56 cc.)	p_H 7·9 Decrease in drop number in 20 mins.	p_H 7·9 Decrease in drop number in 40 mins.	p_H 6·8 Decrease in drop number in 20 mins.	p_H 6·8 Decrease in drop number in 40 mins.
m-Miotine HCl	0	14	25	12	20
	10	0	1	1	1
	1	1	1	5	8
	0·1	0	1·5	9	16
	0·01	8	17	10	18
	0·001	13	24	—	—
m-Miotine MeI	0	9·5	19	9	18
	10	0	1	5·5	11
	1	2	4	8	16
	0·1	7·5	15·5	—	—
HCl of *p*-urethane (benzyl series)	0	10	20	11	20
	10	0	0	0	0
	1	0	0·5	0·5	1
	0·1	1·5	3	9	15
	0·01	8	15	9	17
MeI of *p*-urethane (benzyl series)	0	9·5	18·5	8·5	15·5
	10	0	0	0·5	1·5
	1	1	1·5	2	3·5
	0·1	5	10	8	16
HCl of *m*-urethane (phenyl series)	0	9·5	17·5	7	14·5
	1	1	1	1	1·5
	0·1	1	1·5	0	1
	0·01	0	1	1	2
	0·001	1	3	2	4
	0·0001	4	9	7	14
MeI of *m*-urethane (phenyl series)	0	9	19	8·5	15·5
	10	0	0	2	2
	1	0	0	5	9
	0·1	2	4	—	—
	0·01	7	14	—	—

results. Similar results were obtained with the isomerides of the urethanes mentioned in Table VI; since, however, the same enzyme preparation was not employed with all the compounds, these results do not necessarily indicate correctly the relative inhibitory activities of the various compounds and are not therefore quoted. It should be mentioned in connection with these results that the same enzyme preparation was always employed in the experiments with a given urethane, although, owing to the smaller activity of the esterase at p_H 6·8 a greater volume of the enzyme preparation was used in the more acid solution. Thus in the experiments with the hydrochloride of the methylurethane of *p*-hydroxybenzyldimethylamine in which the enzyme showed the same activity, corresponding with a decrease of 20 in the drop number in 40 minutes, at the two acidities, 33 % more enzyme was employed at p_H 6·8 than at 7·9. In every instance quoted in Table VI the inhibitory activity of a given urethane is smaller at p_H 6·8, *i.e.* on the acid side of neutrality, than at p_H 7·9, and the same effect was consistently observed with the remaining urethanes. As an example, the results with the hydrochloride of the methylurethane of *p*-hydroxybenzyldimethylamine, mentioned above, may be taken,

since identical esterase activities, as shown by the control experiments, were employed at the two acidities. In this case, the amounts of inhibitor required to diminish the activity of the enzyme by 25 %, *i.e.* to diminish the decrease in drop number from 20 to 15, were 0·01 and 0·1 mg. at p_H 7·9 and 6·8 respectively. The inhibitory activity of the urethane at p_H 6·8 was thus only one-tenth of that which it exhibited at p_H 7·9. Ratios of the same order of magnitude were obtained with the other urethanes. To what extent the partial inactivation of the enzyme which takes place in acid solution, and which necessitated the employment of larger volumes of the preparation at p_H 6·8, contributes to this result cannot at present be determined.

Attention should further be directed to the results obtained with the hydrochloride of the methylurethane of *m*-dimethylaminophenol. Reference to Table VI will show that 0·0001 mg. of this substance produced, at p_H 7·9, an inhibition of the activity of the liver esterase of approximately 50 %. Since, in the final reaction mixture, the inhibitor was contained in a volume of about 50 cc., the final concentration of this substance required to diminish the activity of the esterase by this amount was about 1:500,000,000. This is the greatest activity which has been observed with any of the urethanes so far examined. It agrees with other results obtained with the same urethane, which has, in fact, proved to be the most active inhibitor amongst the three series of compounds investigated.

Comparison of the inhibitory activities of isomeric urethanes. In order to obtain a direct comparison of the activities of the members of a given series of urethanes, it was necessary, for reasons stated in connection with the similar experiments carried out with methyl butyrate as substrate, to measure the inhibitory activities on the same enzyme preparation. The results are recorded in Table VII. Different esterase solutions were employed for the three series, so that the figures recorded do not necessarily give a comparison of the activities of members of different series. In the benzyl and phenyl series, an interval of some days elapsed between the experiments with the hydrochlorides and methiodides. The enzyme had meanwhile undergone slight deterioration, as shown by the figures for the second control.

The order of inhibitory activity of the various isomerides on liver esterase, using tributyrin as substrate, is, according to the results of Table VII as follows.

Miotine series. The hydrochlorides do not show large differences in activity. Nevertheless they appear to be definitely in the order $m > o > p$. With the methiodides, which are considerably less active, the *o*-compound exhibits the greatest activity; the *m*- and *p*-isomerides are, within the limits of experimental error, equally active.

Benzyl series. The hydrochlorides of this series show greater differences in activity, the order being quite definitely $o > m > p$. The methiodides again show little difference in activity; the order $p > o > m$ is indicated by the results.

Table VII. *Comparison of the inhibitory activities of isomeric urethanes.*

Substrate: tributyrin. T = 20°. p_H = 7·9.

Inhibitor	Final conc. of inhibitor (molar × 10^{-7})	Decrease in drop number in 20 mins.	Decrease in drop number in 40 mins.	Percentage inhibition
Miotine series:	Control	11	22	—
m-HCl	40	0	2	91
,,	4	5	10	56
p-HCl	40	3·5	7·5	68
,,	4	6	12	47
o-HCl	40	1	3·5	86
,,	4	5	10·5	52
m-MeI	400	2	3	87
p-MeI	400	2	4	83
o-MeI	400	0	1·5	91
Benzyl series:	Control	12	23	—
m-HCl	400	1	2·5	87
,,	40	8	16	30
p-HCl	400	4	8·5	61
,,	40	10	20	13
o-HCl	400	0	1	96
,,	40	3	6	84
	Control	11·5	21	—
m-MeI	400	7	15	29
p-MeI	400	6	12	43
o-MeI	400	6·5	13·5	38
Phenyl series:	Control	13	27	—
m-HCl	4	1	2	90
p-HCl	4	5	10	63
o-HCl	4	5	10·5	59
	Control	11·5	21	—
m-MeI	40	8	16	24
p-MeI	40	8	16	24
o-MeI	40	3	7	67

Phenyl series. In this series the *m*-hydrochloride is considerably more active than the *o*- and *p*-compounds, which can scarcely be distinguished from one another in activity. Of the methiodides, the *o*-compound is the most active, the *m*- and *p*-isomerides possessing the same activity. It should be noted that the order of inhibitory activity of both the hydrochlorides and methiodides of this series is identical with that of the miotine series.

Discussion.

The foregoing results have established definitely that urethanes of the type which have been shown in earlier papers to possess very specific physiological properties also possess the common property of inhibiting in low concentration the activity of liver esterase. Of the nine urethanes examined, all exhibit this property to a marked degree, and not only do they do so in the form of their hydrochlorides but their methiodides are similarly, although usually somewhat less, active. It appears legitimate to conclude that a relationship exists between chemical constitution and inhibitory activity towards liver esterase analogous to that between constitution and physiological action in the same group. In conformity with this conclusion, the methylurethane of choline

iodide, a substance containing many structural features in common with the active urethanes but lacking the essential phenyl group, is inactive. Similarly, the hydrochloride of *α-m*-hydroxyphenylethyldimethylamine, the phenol from which miotine is prepared and which contains the phenyl and basic but not the urethane group, produces no inhibition in relatively high concentration.

Whether these results hold for the enzyme, present in blood-sera from certain species and in tissue extracts, which is normally responsible for the destruction of acetylcholine, has not yet been determined. Nevertheless, the fact that urethanes of the type which possess physiological properties similar to those of physostigmine also inhibit, in minute concentration, the hydrolysis by liver esterase of a simple ester such as methyl butyrate, affords substantial support to the view that the above-mentioned serum-enzyme, the activity of which towards acetylcholine has been shown by Loewi and his co-workers and by Matthes to be inhibited by physostigmine, is a true esterase such as is present in the liver and is not one which acts specifically towards acetylcholine.

It is not proposed to discuss in detail the question, which now arises, as to whether the physiological activity of urethanes of the miotine type is an indirect result of the inhibitory action which they exert upon esterases, since this problem has already been considered from many aspects by White and Stedman [1931], who have pointed out that while much of the activity of miotine, in particular its toxicity, may probably be attributed to its inhibition of the destruction of acetylcholine, other mechanisms are possibly also involved. One fact, which supports the suggestion, originally made by Loewi and Navratil [1926], that the action of physostigmine on the heart is due to its inhibitory action on the acetylcholine-destroying enzyme, may, however, be mentioned. It has been shown above that arecoline, in relatively high concentration, does not inhibit the activity of liver esterase. Now this alkaloid belongs, pharmacologically, to the physostigmine group of drugs and might therefore be expected to resemble physostigmine in its behaviour towards esterases. That it does not do so is in complete accord with some unpublished pharmacological experiments by Dr A. C. White, to whom we are indebted for information regarding his results, according to which arecoline, unlike physostigmine, does not potentiate the action of acetylcholine on the vagus.

It is not to be expected, even on the assumption that the whole of the physiological activity of urethanes of the miotine type is attributable to the resulting accumulation of acetylcholine in the organism, that an exact parallelism will exist between the magnitude of their physiological activities and that of their inhibitory activities towards esterases, for the drugs will be subject to many influences in the organism which are eliminated in *in vitro* experiments with enzymes. Thus, a property of a drug, such as adsorbability, which may render it particularly efficient in inhibiting the activity of an esterase, may actually prevent it from reaching the enzyme in the organism. Similarly, stability will be of greater influence in *in vivo* than in *in vitro* experiments, since in the former case the drug is subjected to the action of

more destructive agents. That no such parallelism does, in fact, exist follows from our experiments; the order of the inhibitory activity of various urethanes towards liver esterase in no way corresponds with the previously published provisional order for their miotic activities.

Perhaps the most interesting questions which call for consideration in connection with our experiments are those concerning the nature of the inhibitory action and its bearing on the mechanism of the normal action of esterases. Of a group of compounds possessing a common structural feature, every member which has been examined has been found to inhibit, in minute concentration, the activity of liver esterase. These inhibitory substances, like the normal substrate of the enzyme, are esters. Nevertheless they are either not attacked, or are only hydrolysed extremely slowly, by the esterase. The suggestion thus arises that the urethanes combine with the esterase in the same way and by means of the same mechanism as do simple esters, but that the affinity between urethane and enzyme is enormously greater than that between simple ester and enzyme. Hence the esterase-urethane compound is formed preferentially and, owing to the inability of the enzyme to decompose the urethane, the hydrolysis of the normal substrate is prevented. The latent period which occurs in the hydrolysis of ethyl mandelate by liver esterase when esters of certain keto-acids are present has been similarly explained by Willstätter, Kuhn, Lind and Memmen [1927], and further experiments in support of this explanation have been published by Bamann and Schmeller [1930]. On the above basis the relative inhibitory activities of the various urethanes will be a measure of their affinities for the esterase, and any regularity which is observed between inhibitory and other properties should give a clue as to the nature of the affinity between the urethanes and esterase, and hence of the nature of the forces normally responsible for the formation of enzyme-substrate compound. Now, the following regularities have, in fact, been noted in our experiments.

(1) In a given series of urethanes the isomerides show inhibitory activities which, although usually different, are of the same order of magnitude.

(2) Of the three series examined, the hydrochlorides of the phenyl series are outstanding with respect to their high inhibitory activities.

(3) The methiodides are, in general, considerably less active than the hydrochlorides of the corresponding tertiary bases.

(4) The inhibitory activity of a given urethane is considerably smaller at p_H 6·8 than in slightly alkaline medium.

We believe that these results can be best interpreted by assuming that the affinities in question are, in the first instance, of the nature of adsorptive forces. If we postulate (*a*) that the urethane group confers high adsorbabilities on the inhibitory substances, (*b*) that the adsorbability and therefore inhibitory activity is increased by any factor which tends to lower the solubility, (*c*) that the free bases and not the salts are the active inhibitors, our reasons for this belief will be at once clear. In accordance with postulates (*b*) and (*c*),

any factor which tends to convert the readily soluble salts into the sparingly soluble bases, thus diminishing the solubility and increasing the concentration of the active constituent, would be expected to assist adsorption and hence increase inhibitory activity. Increase in alkalinity is such a factor, and as shown above this increases the inhibitory activity. Similarly diminishing the basic strength of the compounds would constitute another such factor; corresponding with this the compounds which are the weakest bases, namely, the methylurethanes of the phenyl series, show the greatest inhibitory activity. Further, solubility is increased by converting the tertiary bases into their quaternary ammonium salts, the free bases of which are readily soluble in water. This should diminish adsorbability and hence inhibitory activity, as is actually the case.

Nevertheless, adsorption is clearly not the only process involved. The possession of surface activity of a high order will not necessarily confer upon a compound the marked inhibitory activity shown by the above urethanes. Moreover, it has been shown by Willstätter, Kuhn and Bamann [1928] that esters of *d*- and *l*-mandelic acids exhibit different affinities towards esterases, while Murray and King [1930] have demonstrated that certain enantiomorphic alcohols inhibit the activity of liver esterase to different degrees. Optical isomerides would be expected to possess identical adsorbabilities; it is therefore almost certain that some factor other than adsorption is also involved. We are of opinion that, following adsorption, the inhibitor enters into especial relation with the enzyme, the exact nature of which is not at present clear, and it is probably this second stage of the combination process which is the cause of the different inhibitory activities of isomeric urethanes. One fact which has been elicited in our experiments tends to support this view. In examining the inhibitory activities of the various urethanes towards liver esterase, it has been found that the general effect of those factors which we have interpreted above as exerting their influence by producing changes in adsorbability is the same whether the substrate is methyl butyrate or tributyrin. The relative activities of isomeric urethanes vary, however, with the nature of the substrate, a result which cannot be explained on the basis of adsorption.

Summary.

1. Urethanes of the type which have previously been shown to possess physiological properties of the same kind as physostigmine have been found to possess the common property of inhibiting the activity of liver esterase. A relationship thus exists between chemical constitution and inhibitory action towards liver esterase analogous to that between constitution and physiological activity in the same group.

2. This inhibitory action is exerted in high dilution. Thus, the hydrochloride of the *m*-dimethylaminophenyl ester of methylcarbamic acid, which

is the most active urethane examined, produced an inhibition of 50 % in the hydrolysis of tributyrin by liver esterase when in a final concentration of about 1:500,000,000. The same urethane inhibited the hydrolysis of methyl butyrate to about the same extent when in a final concentration of $\frac{1}{3} \times 10^{-6}$ *M*, which corresponds with one of about 1:13,000,000.

3. Three series of isomeric urethanes, which have been termed the methylurethanes of the phenyl, benzyl and miotine series respectively, have been examined for inhibitory activity in the forms both of their hydrochlorides and methiodides. The relative activities of the isomerides, when methyl butyrate is employed as substrate, are as follows.

Hydrochlorides. Phenyl series: $m > p > o$; benzyl series: $m > p > o$; miotine series: $m = p > o$.

Methiodides. Phenyl series: $m \geqq p > o$; benzyl series: $p > o > m$; miotine series: $o > p \geqq m$.

Using tributyrin as substrate the following orders, which differ from those above, are obtained:

Hydrochlorides. Phenyl series: $m > p \geqq o$; benzyl series: $o > m > p$; miotine series: $m > o > p$.

Methiodides. Phenyl series: $o > p = m$; benzyl series: $p > o > m$; miotine series: $o > p = m$.

4. The inhibitory activity is, in every case, greater in slightly alkaline medium than at p_H 6·8.

5. In general, the methiodides exert a considerably smaller inhibitory effect than do the hydrochlorides.

6. The relative inhibitory activities of the various urethanes do not correspond with their relative miotic activities. It is pointed out that such correspondence is not to be expected even on the assumption that the whole of the physiological activity of the urethanes is due to their inhibitory action on esterases.

7. The mechanism of the inhibitory action is discussed.

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CXXX. THE GLYCERIDE STRUCTURE OF BEEF TALLOWS.

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In continuation of the studies of the constitution of different classes of natural fats which have occupied our attention for some time, an examination has been made of four samples of beef tallow which varied in composition within fairly wide limits. With the exception of a specimen of mutton tallow [Collin, Hilditch and Lea, 1929], body fats from the ox or sheep have not hitherto been submitted to complete investigation by the present methods.

Bömer and his co-workers [1907, 1909], by repeated fractional crystallisation of the solid glycerides present in tallow, established the presence of a number of simple and mixed glycerides. Thus, these workers state that beef tallow contains 0·5–1·5 % of tristearin and varying proportions of α-palmitodistearin, stearodipalmitin, oleodipalmitin and oleopalmitostearin, and that about 3 % of tristearin, 4–5 % of α-palmitodistearin, 4–5 % of stearodipalmitin, were accompanied in mutton tallow by larger quantities of oleodipalmitin and oleopalmitostearin. In the specimen of mutton tallow investigated by the present methods, Collin, Hilditch and Lea [1929] found 26 % of fully-saturated compounds which consisted for the most part of mixed glycerides of palmitic and stearic acid; fractional crystallisation of the fully-saturated components indicated the probable presence of about 3 % of tristearin, 9 % of palmitodistearin and 6–7 % of dipalmitostearin, leaving about 7–8 % of the original fat to be accounted for as tripalmitin and mixed glycerides of myristic, palmitic and stearic acids.

In the present work we have not carried out any fractional crystallisation of the solid components of the tallows, since we desire rather to discuss the general composition of the fats from a quantitative standpoint, in a similar manner to the recent treatment of the component glycerides of butter fat by Hilditch and Sleightholme [1931].

The beef tallows employed in the present work were from American consignments kindly selected for us by Messrs Lever Bros., Ltd., and consisted of a North American tallow (N) and three samples of South American premier jus which will be referred to respectively as SA, SB and SC. Each material thus represents bulk samples typical of different centres of production rather than fat from a single animal or even from separate herds. This was desirable from the original motive of this particular investigation, which was to establish

the main characteristics of market supplies of beef tallows. As the work progressed certain general features became apparent, *viz.* that, as in the butter fats [Hilditch and Sleightholme, 1931], (i) the composition of the fully-saturated glycerides tends to be constant irrespective of their proportion in the whole fat, and (ii) the combined amounts of stearic, oleic and linoleic acids in the whole fats also tend towards a constant value, irrespective of wide variations in the respective amounts of stearic and of unsaturated (oleic and linoleic) acids.

The saponification equivalents and iodine values of the specimens examined were as follows:

Table I.

Beef tallow	M.P. (open tube)	Saponification equivalent	Iodine value	Setting-point of mixed fatty acids
N	42°	284·2	49·5	42·3°
SA	46°	280·5	42·1	45·9°
SB	47·5°	284·0	37·1	47·2°
SC	47·5°	285·7	39·3	47·0°

Component fatty acids of the four beef tallows.

The composition of the mixed fatty acids from the tallows was determined according to the methods which we have described on several occasions in some detail [*cf.* Hilditch and Jones, 1929, 1931]. The mixture of fatty acids (palmitic, stearic, oleic with minor amounts of myristic and linoleic) is comparatively simple in this case, and, after preliminary separation into the usual groups of "solid" and "liquid" acids by the lead salt and alcohol separation, the fractionation of the corresponding group of methyl esters presents no great difficulty. The final results of the analyses only are tabulated below but, in order to illustrate the method of calculation employed, the fractionation data for one of the tallows (N) are reproduced in Table II.

In calculating the results, it is assumed (i) that the only unsaturated acid present in the esters of the "solid" acids is oleic, and (ii) that the mixture of unsaturated esters distilling over throughout from the esters of the "liquid" acids has the same composition as that of the pure unsaturated ester fraction (L 8 in Table II) finally obtained. The mean saponification equivalents of the saturated esters in each fraction are determined after making allowance for those of the unsaturated esters present.

Some trouble was experienced with the onset of rancidity in the original fats and, by the time specimens SB and SC were analysed, this had developed

Table II. *Fatty acids of beef tallow N.*

	Lead salt separation.		Corresponding esters	
	g.	%	Sap. eq.	I.V.
"Solid" acids S	113	46·5	278·2	7·0
"Liquid" acids L	130	53·5	287·5	80·71
	243			

Table II (*contd.*).

Fractionation and estimated composition of methyl esters.

(i) *Esters of "solid" acids S.*

No.	g.	B.P./1 mm.	Sap. eq.	I.V.	C_{14}	C_{16}	C_{18}	Ol.
S 1	4·68	114 –129°	264·3	2·7	1·00	3·53	—	0·15
S 2	6·24	129 –128	265·9	3·0	1·00	5·02	—	0·22
S 3	5·47	128 –130	268·1	3·2	0·47	4·80	—	0·20
S 4	9·53	130 –132	270·5	3·8	0·14	8·97	—	0·42
S 5	9·53	132 –132·5	271·5	4·1	—	8·79	0·29	0·45
S 6	10·24	132·5–133	275·5	4·6	—	8·00	1·69	0·55
S 7	10·10	133 –136	278·1	5·8	—	6·89	2·53	0·68
S 8	10·07	136	280·8	7·3	—	5·91	3·30	0·86
S 9	9·76	136 –138	284·4	9·6	—	4·43	4·24	1·09
S 10	8·57	138 –141	289·6	11·7	—	2·32	5·08	1·17
S 11	11·19	141 –146	295·8	13·0	—	0·65	8·85	1·69
S 12	5·37	Residue	297·5	14·8	—	0·06	4·38	0·93
				Weights	2·61	59·37	30·36	8·41
				% esters	2·6	59·0	30·1	8·3
				% acids	2·6	58·8	30·2	8·4

(ii) *Esters of "liquid" acids L.*

Primary fractions

No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 1	36·94	71–137·5°	273·8	69·4
L 2	11·63	137·5	285·5	82·5
L 3	14·11	137·5–144	291·7	85·0
L 4	9·11	144	292·9	86·3
L 5	9·55	144–145	292·6	86·3
L 6	10·46	145–144	293·8	88·2
L 7	8·88	144	294·0	89·0
L 8	8·58	144–147	296·3	90·2
L 9	8·65	Residue	320·6	88·6

Refractionations

No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 11	3·34	73–105°	244·5	40·7
L 12	5·68	105–110	256·1	53·0
L 13	3·53	110–118	270·1	64·8
L 14	3·45	118–128	279·4	72·8
L 15	3·97	128–130	284·0	76·3
L 16	3·64	130–135	288·2	79·9
L 17	3·27	135	290·2	83·4
L 18	4·52	Residue	291·6	86·6

No.	g.	as C_{14}	C_{16}	Oleic	Linoleic	Unsap.
L 1	36·94	8·58	—	28·36		—
L 2	11·63	0·99	—	10·64		—
L 3	14·11	0·82	—	13·29		—
L 4	9·11	0·25	0·14	8·72		—
L 5	9·55	0·42	—	9·13		—
L 6	10·46	0·23	—	10·23		—
L 7	8·88	0·12	—	8·76		—
L 8	8·58	—	—	8·58		—
L 9	8·65	—	—	7·99		0·66
Weights		11·41	0·14	100·33	5·37	0·66
% esters		9·7	0·1	85·1	4·6	0·5
% acids		9·6	0·1	85·2	4·6	0·5

No.	g.	as C_{14}	Oleic	Linoleic
L 11	3·34	1·83	1·51	
L 12	5·68	2·34	3·34	
L 13	3·53	0·99	2·54	
L 14	3·45	0·67	2·78	
L 15	3·97	0·61	3·36	
L 16	3·64	0·42	3·22	
L 17	3·27	0·25	3·02	
L 18	4·52	0·18	4·34	
Weights		7·29	24·11	
% esters		23·2	76·8	

to an appreciable extent. In consequence the values obtained for the linoleic acid contents of these fats are almost certainly lower, and those for myristic acid higher, than the truth owing to the presence of traces of low-boiling products of decomposition which it is not yet possible accurately to correct for in the course of the analysis. A summary of the analytical data obtained for the mixed fatty acids from each tallow is given in Table III.

Table III. *Summarised experimental data for fatty acids of beef tallows.*

Acid	Solid acids S	Liquid acids L	Total	Fatty acids (excluding unsaponifiable matter) % (wt.)	(% mols.)
		Beef tallow N.			
	(46·5 %)	(53·5 %)			
Myristic	1·20	5·12	6·32	6·3	7·5
Palmitic	27·35	0·06	27·41	27·4	29·1
Stearic	14·06	—	14·06	14·1	13·4
Oleic	3·89	45·57	49·46	49·6	47·6
Linoleic	—	2·44	2·44	2·5	2·4
Unsaponifiable	—	0·31	0·31	—	—
		Beef tallow SA.			
	(54·0 %)	(46·0 %)			
Myristic	0·43	4·03	4·46	4·5	5·3
Palmitic	30·56	—	30·56	30·6	32·9
Stearic	19·04	—	19·04	19·1	18·1
Arachidic	0·12	—	0·12	0·1	0·1
Oleic	3·85	38·78	42·63	42·7	40·7
Linoleic	—	2·96	2·96	3·0	2·9
Unsaponifiable	—	0·23	0·23	—	—
		Beef tallow SB.			
	(59·0 %)	(41·0 %)			
Myristic	1·83	6·00	7·83	7·8	9·5
Palmitic	27·70	—	27·70	27·8	29·2
Stearic	24·37	—	24·37	24·4	23·2
Oleic	5·10	33·67	38·77	38·9	37·1
Linoleic	—	1·08	1·08	1·1	1·0
Unsaponifiable	—	0·25	0·25	—	—
		Beef tallow SC.			
	(57·7 %)	(42·3 %)			
Myristic	1·26	4·40	5·66	5·8	6·9
Palmitic	23·68	—	23·68	24·0	25·5
Stearic	28·22	—	28·22	28·6	27·4
Oleic	4·54	36·48	41·02	41·6	40·2
Unsaponifiable	—	1·42	1·42	—	—

Fully-saturated glyceride contents of the four beef tallows.

Each fat was submitted, in quantities of 100 g. at a time, to oxidation in ten times its weight of acetone by means of four times its weight of powdered potassium permanganate. The procedure has been recently described in detail by Hilditch and Sleightholme [1931]. The results obtained are given in detail in Table IV.

Table IV. *Determination of fully-saturated glyceride (F.S.G.) content.*

(i) *Analytical data.*

			Purification of crude fully-saturated glycerides							
				"A"		"B"		Acidic products		
No.	Weight taken g.	Crude F.S.G. g.	Weight taken g.	g.	Acid No.	g.	Acid No.	g.	Acid No.	F.S.G. % (wt.)
SB	399·8	117·5	115·6	85·6	0·5	15·7	28·5	14·3	270·0	25·3
SC	204·4	56·0	54·7	42·9	0·4	9·2	25·1	2·6	232·7	25·6
SA	316·0	86·4	84·8	39·4	0·7	32·9	22·3	12·2	278·9	22·5
N	604·2	92·0	81·0	39·5	0·8	35·2	10·4	6·3	167·7	13·6

(ii) *Summary of observed values.*

		Acids in whole fat		Fully-saturated glycerides		"Association ratio" in non-fully-saturated part.
No.	Iodine value	Saturated % (mol.)	Unsaturated % (mol.)	Weight %	Mols. %	Mols. saturated acid per mol. unsaturated acid
SB	37·1	61·9	38·1	25·3	25·8	0·94
SC	39·3	59·8	40·2	25·6	26·0	0·84
SA	42·1	56·4	43·6	22·5	22·8	0·75
N	46·6	50·0	50·0	13·6	13·9	0·72

The purified fully-saturated glycerides "A" (*cf.* Table IV) were converted into their mixed fatty acids, and thence into the corresponding mixed methyl esters, which were fractionally distilled in the usual manner. Since these analytical results have led us to conclude that there is a considerable approach to constancy in the composition of the fatty acids of the fully-saturated components of the tallows, it may be well to record the fractionation data in full (Table V).

Table VI illustrates the general composition of 100 parts by weight of the four tallows for the whole fats, for the fully-saturated glyceride portions, and (by difference) for the portions of the fat consisting of mixed saturated-unsaturated and (possibly) fully-unsaturated glycerides.

Discussion.

Before proceeding to consider the fatty acids present in the whole tallows and in their component subdivisions of fully-saturated and non-fully-saturated glycerides, attention may be directed to the general relationships subsisting between the fully-saturated glyceride content and the total content of saturated acids in each tallow. The range of unsaturation encountered in these fats is not relatively so great as that which was available in the series of butter fats [1931], but nevertheless it suffices, we believe, to show that the same kind of relationship subsists in the beef tallows as in cow milk fats. Moreover, the data formerly obtained [Collin, Hilditch and Lea, 1929] for a mutton tallow also display the same general features and, although we do not wish to minimise the differences which in many cases characterise fats from different species or genera of animals or plants, the body fats of the sheep and ox

Table V. *Fractionation of methyl esters from fully-saturated glycerides.*

Fraction	g.	B.P./1 mm.	Sap. eq.	Myristate g.	Palmitate g.	Stearate g.	Unsap. g.
			Fully-saturated glycerides of beef tallow SB.				
1	3·68	121–131°	262·4	0·92	2·76	—	—
2	4·32	131–132	266·8	0·45	3·87	—	—
3	4·28	132–134	272·4	—	3·87	0·41	—
4	3·23	134–140	277·3	—	2·33	0·90	—
5	6·20	140–141	279·6	—	3·93	2·27	—
6	8·26	141–145	286·1	—	3·31	4·95	—
7	5·25	145	289·0	—	1·58	3·67	—
8	5·75	Residue	304·3	—	0·77	4·78	0·20
	40·97		Weights	1·37	22·42	16·98	0·20
			% esters	3·3	54·7	41·5	0·5
			% acids	3·3	54·6	41·6	0·5
			Fully-saturated glycerides of beef tallow SC.				
1	3·76	104–128°	263·0	0·86	2·90	—	—
2	4·44	128–130	268·1	0·27	4·17	—	—
3	4·88	130–135	274·1	—	4·10	0·78	—
4	3·79	135–137	280·7	—	2·25	1·54	—
5	3·34	137–140	286·3	—	1·32	2·02	—
6	3·27	140	289·8	—	0·89	2·38	—
7	5·32	Residue	305·7	—	0·47	4·67	0·18
	28·80		Weights	1·13	16·10	11·39	0·18
			% esters	3·9	55·9	39·6	0·6
			% acids	3·9	55·8	39·7	0·6
			Fully-saturated glycerides of beef tallow SA.				
1	1·43	104–120°	247·8	1·11	0·32	—	—
2	1·55	120–129	259·5	0·54	1·01	—	—
3	3·31	129–125	266·6	0·36	2·95	—	—
4	2·85	125–123	271·6	—	2·67	0·18	—
5	2·52	123–119	275·8	—	1·96	0·56	—
6	2·94	119–116	282·8	—	1·52	1·42	—
7	10·21	Residue	294·6	—	3·41	6·57	0·23
	24·81		Weights	2·01	13·84	8·73	0·23
			% esters	8·1	55·8	35·2	0·9
			% acids	8·0	55·7	35·3	1·0
			Fully-saturated glycerides of beef tallow N.				
1	2·39	104–117°	258·3	0·94	1·45	—	—
2	3·65	117–124	265·0	0·60	3·05	—	—
3	3·06	124–127	272·6	—	2·75	0·31	—
4	2·94	127–132	277·0	—	2·15	0·79	—
5	3·67	132–136	281·7	—	2·05	1·62	—
6	4·07	136–140	287·2	—	1·48	2·59	—
7	5·05	Residue	309·1	—	0·38	4·45	0·22
	24·83		Weights	1·54	13·31	9·76	0·22
			% esters	6·2	53·6	39·3	0·9
			% acids	6·2	53·5	39·4	0·9

happen not to be very dissimilar in composition and it may be useful to include the data for the specimen of sheep tallow formerly examined[1].

In the case of the butter fats we showed that there was a parallelism between the molar proportion of fully-saturated glycerides and (i) the proportion of saturated and unsaturated acids in the whole fat, and (ii) the corresponding molar proportion of fully-saturated glycerides in synthesised

[1] The mutton tallow is designated in the tables by (M).

Table VI. *General composition (weights) of beef tallows.*

	Whole fat	Fully-saturated	Non-fully-saturated
	Beef tallow SB.		
	100 g.	25·3 g.	74·7 g.
Glyceryl residue $C_3H_2\equiv$	4·45	1·15	3·3
Myristic acid	7·45	0·8	6·65
Palmitic ,,	26·55	13·25	13·3
Stearic ,,	23·3	10·1	13·2
Oleic ,,	37·2	—	37·2
Linoleic ,,	1·05	—	1·05
	Beef tallow SC.		
	100 g.	25·6 g.	74·4 g.
Glyceryl residue $C_3H_2\equiv$	4·4	1·2	3·2
Myristic acid	5·5	1·0	4·5
Palmitic ,,	23·0	13·7	9·3
Stearic ,,	27·3	9·7	17·6
Oleic ,,	39·8	—	39·8
	Beef tallow SA.		
	100 g.	22·5 g.	77·5 g.
Glyceryl residue $C_3H_2\equiv$	4·5	1·0	3·5
Myristic acid	4·3	2·0	2·3
Palmitic ,,	29·2	11·7	17·5
Stearic ,,	18·3	7·8	10·5
Oleic ,,	40·8	—	40·8
Linoleic ,,	2·9	—	2·9
	Beef tallow N.		
	100 g.	13·6 g.	86·4 g.
Glyceryl residue $C_3H_2\equiv$	4·4	0·6	3·8
Myristic acid	6·0	0·8	5·2
Palmitic ,,	26·3	7·0	19·3
Stearic ,,	13·5	5·2	8·3
Oleic ,,	47·4	—	47·4
Linoleic ,,	2·4	—	2·4

mixed glycerides of corresponding total unsaturation, or (iii) of the molar proportion of fully-saturated glycerides calculated on the basis of proportionality to the cube of the total saturated acids present. At the same time the "association ratio" of saturated and unsaturated acids in the non-fully-saturated glycerides varied correspondingly with the total unsaturation. Similar relationships for the four beef tallows and the mutton tallow (M) are shown graphically in Fig. 1, in which the appropriate proportions of the synthetic glyceride curve (unbroken line) of Bhattacharya and Hilditch [1930] and of the "calculated" curve (dotted) are plotted together with the observed values for the tallows.

This group of tallows therefore affords further confirmation of the generalisation that in animal fats the nature of the mixed glycerides is mainly determined by the relative proportions of saturated and unsaturated acids present. In the body fats and milk fats of cattle, for any given ratio of saturated to unsaturated acids, the mode of assemblage in the triglyceride molecules appears to follow a more or less fixed course with the general result that the

amount of fully-saturated glycerides is somewhat greater than that proportionate to the cube of the total saturated acid content.

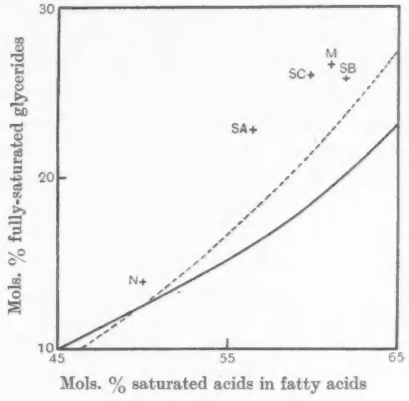

Fig. 1.

The molar association ratios lead to limiting values for the possible proportions of either mono-unsaturated-disaturated and diunsaturated-monosaturated glycerides, or mono-unsaturated-disaturated and tri-unsaturated glycerides which may be present, and the approximate data in question are collected in Table VII. The actual proportion of tri-unsaturated glycerides present is uncertain; the presence of tristearin and (probably) of tripalmitin to a total extent of perhaps 5–8 % of the whole fat has been indicated by fractional crystallisation of the mixed glycerides (*cf.* p. 1168). This indicates a somewhat greater tendency to the production of simple triglycerides than in the butter fats, and, by analogy, there may be some likelihood of the presence of triolein (in amount not exceeding, perhaps, some 10 % of the whole fat). No quantitative means for the determination of triolein being yet available, it is not possible to do more than assign the extreme possible limits of the three classes of unsaturated glycerides quoted in the Table.

Table VII. *Limiting values for the molar percentages of the four types of mixed glycerides in the fat.*

			Glycerides (mols. %)			
Tallow	Iodine value	"Association ratio"	Fully-saturated	Mono-unsaturated-disaturated	Diunsaturated-mono-saturated	Tri-unsaturated
SB	37·1	0·94	25·8	33·7–53·9	40·5–0	0–20·3
(M)	41·2	0·90	26·6	30·9–52·1	42·5–0	0–21·3
SC	39·3	0·84	26·0	27·3–50·7	46·7–0	0–23·3
SA	42·1	0·75	22·8	22·1–49·6	55·1–0	0–27·6
N	49·5	0·72	13·9	22·0–54·1	64·1–0	0–32·0

Distribution of individual fatty acids in the glycerides of tallows.

The discussion of the component fatty acids of the tallows will be based throughout on the proportionate number of molecules of the different fatty acids present, that is, on the molar percentages (and not the weight percentages) of the fatty acids. In Table VIII will be found respectively the molar distribution of fatty acids (1) in the whole tallows, (2) in their fully-saturated glycerides, and (3) in their mixed saturated-unsaturated glycerides; the fats are conveniently dealt with in decreasing order of fully-saturated glyceride content.

Table VIII.

	(M)	SC	SB	SA	N
Molar distribution of fatty acids.					
(1) Whole fat:					
Myristic acid	5·5	6·9	9·5	5·3	7·5
Palmitic „	26·2	25·5	29·2	32·9	29·1
Stearic „	29·3	27·4	23·2	18·2	13·4
Oleic „	34·8	40·2	37·1	40·7	47·6
Linoleic „	4·2	—	1·0	2·9	2·4
(2) Fully-saturated glycerides:					
Molar % F.S.G.	26·6	26·0	25·8	22·8	13·9
Myristic acid	7·1	4·5	3·9	9·3	7·2
Palmitic „	52·1	58·2	57·0	57·7	55·8
Stearic „	40·8	37·3	39·1	33·0	37·0
(3) Mixed saturated-unsaturated glycerides:					
Molar % M.S.-U.	73·3	74·0	74·2	77·2	86·1
Myristic acid	4·9	7·8	11·1	3·7	7·5
Palmitic „	16·9	13·9	19·8	25·4	24·8
Stearic „	25·0	23·9	17·7	13·7	9·6
Oleic „	47·5	54·4	50·0	53·4	55·3
Linoleic „	5·7	—	1·4	3·8	2·8

As in the case of the butter fats, considerable interest attaches to comparison of the proportion of C_{18} (stearic, oleic and linoleic) acids present with the total proportion of the remaining acids (palmitic with small amounts of myristic). The molar percentages of these groups present in each fat and in its two subdivisions are shown in Table IX.

Table IX. *Molar distribution of myristic-palmitic and C_{18} acids in the tallows.*

Fat ...		(M)	SC	SB	SA	N
Section	Acids					
Whole fat	Myristic-palmitic	31·7	32·4	38·7	38·2	36·6
	C_{18} acids	68·3	67·6	61·3	61·8	63·4
	(Stearic	29·3	27·4	23·2	18·2	13·4)
Fully-saturated	Myristic-palmitic	59·2	62·7	60·9	67·0	63·0
	Stearic	40·8	37·3	39·1	33·0	37·0
Saturated-unsaturated	Myristic-palmitic	21·8	21·7	30·9	29·1	32·3
	C_{18} acids	78·2	78·3	69·1	70·9	67·7
	(Stearic	25·0	23·9	17·7	13·7	9·6)
	(Oleic-linoleic	53·2	54·4	51·4	57·2	58·1)

Component fatty acids of the whole fats.

Table VIII (1) illustrates the general composition of the fatty acids of the tallows which we have studied, and shows that oleic, palmitic and stearic acids are the predominating components, accompanied by relatively small amounts of linoleic acid and of myristic acid[1]. The values for palmitic acid are the most constant in the group, and in this respect resemble the corresponding data for palmitic acid in butter fats; indeed, they suggest, like those of the butter fats, that palmitic acid on the one hand and the C_{18} acids on the other probably have little in common so far as their mode of synthesis in the plant or animal is concerned.

Our meaning will perhaps be clearer if Table IX is consulted, and the molar percentages of the C_{18} acids in the whole tallows are considered. The only remaining acids are palmitic and myristic, which are given together (palmitic acid accounting for 70 %, or usually more, of this group). The five specimens now under consideration (including the mutton tallow M) fall into two clearly defined groups when classified in this way: specimens M and SC contain about 68 mols. % of C_{18} acids, and the remaining three about 61–63 mols. %, although throughout the five fats the molar proportion of saturated C_{18} (stearic) acid varies from 13 to 29 mols. %. Taking the group as a whole, the total content of C_{18} acids thus tends much more towards a more or less steady value than does the content of the stearic acid included therein.

It is interesting to add to the foregoing data the following earlier analyses of tallow mixed fatty acids given by Armstrong and Allan [1924]. Their figures, reduced to molar percentages as in Tables VIII and IX, are quoted in Table X.

Table X.

[From Armstrong and Allan, 1924.]

	Beef			Mutton	
	North American	South American	Australian	South American	Australian
Myristic acid	2·4	3·0	2·4	1·8	2·4
Palmitic ,,	34·5	26·7	28·3	22·5	26·8
Stearic ,,	13·9	19·3	21·7	29·0	22·2
Oleic ,, / Linoleic ,,	49·2	51·0	47·6	46·7	48·6
Myristic-palmitic	36·9	29·7	30·7	24·3	29·2
C_{18} acids	63·1	70·3	69·3	75·7	70·8
(Stearic	13·9	19·3	21·7	29·0	22·2)

It will be seen that, of these five specimens, three contain 69–71 % of C_{18} acids and one 63 %, whilst the fifth sample is definitely out of line with either group.

Thus out of nine samples of beef and mutton tallows which have been submitted to detailed analysis five contain about 70 mols. % of C_{18} acids with

[1] It should be recalled that in specimens SB and SC the myristic acid figures given are probably slightly high, and those for linoleic acid somewhat low, owing to the onset of rancidity as mentioned on p. 1171.

about 30 mols. % of palmitic (with myristic) acid, and four contain about 62 % of C_{18} acids with about 38 % of palmitic (with myristic) acid—the stearic acid molar content meantime varying from 13 to 29 %. Whilst much further study of tallows from specific herds or animals would probably be necessary to settle the point, there is some indication here that two classes of mixed fatty acids may occur, in both of which the relative amounts of C_{18} and the remaining acids are almost constant; although relative proportions vary somewhat between one group and the other, both are marked by a characteristic proportion of total C_{18} acids irrespective of wide variations in the subordinate proportions of saturated (stearic) and unsaturated (oleic with linoleic) acids.

The constitution of the tallow mixed glycerides.

If the component fatty acids of the fully-saturated parts of the fats are considered under the headings of stearic and palmitic (with myristic), as in Table IX, it will be observed that the molar percentage of stearic acid lies between 37 and 41 in the case of four out of the five tallows, the remaining one (SA) being slightly lower (33). On comparing these values for stearic acid with those in the whole fats, it is evident that the composition of the fully-saturated glycerides tends to be much the same however greatly the proportion of stearic acid present in the whole of the fat varies (13–29 mols. %), and whatever the fully-saturated glyceride content (14–26 %) of the fat[1].

This tendency towards constant composition of the fully-saturated portions of the fat also appeared very clearly in the parallel investigation of butter fats [Hilditch and Sleightholme, 1931], and we conclude that the component fatty acids of the fully-saturated part of an animal fat are to a large extent characteristic and definite for a given species.

It is also of some interest to note that the molar proportion of palmitic (with myristic) acid in one group of the whole tallows (37–38 %) is much the same as that recently observed in a number of butter fats (34–37 %), whilst in the corresponding fully-saturated glycerides the usual figure in the tallows appears to be about 60 % as compared with 55–58 % for the milk fats. It may further be observed that, whereas, as already mentioned, the C_{18} acid molar content of the whole tallows seems to lie in one of two groups (*ca.* 62 % and *ca.* 68 %), this distinction disappears in their fully-saturated glycerides, where the usual range is 37–41 %.

So far as the non-fully-saturated glycerides are concerned, it follows arithmetically that the molar contents of the two groups of acids, palmitic (with myristic) and total C_{18}, will show the same resemblance as those in the whole fats: namely, two groups in which, within narrow limits, approximate constancy is again observed (palmitic with myristic acid respectively *ca.* 22 and *ca.* 30—total C_{18} acids respectively *ca.* 78 and *ca.* 70 %). The variation

[1] The fully-saturated glycerides isolated from the four beef tallows melted respectively at 54° (SA), 54·5° (SB), 54·5° (SC) and 54° (N).

in the stearic acid molar content (9·6–25·0 %) is, of course, more pronounced than in the fats as a whole since differences in the amount of stearic acid in the whole tallows are concentrated entirely in the non-fully-saturated portion of the fats.

This feature is perhaps rendered more clear by tabulating the relative proportions of stearic and palmitic (with myristic) acids in the whole fats and in the non-fully-saturated portions, and comparing the percentage values obtained (Table XI) with those for the fully-saturated glycerides in Table IX:

Table XI.

Relative proportions of myristic-palmitic and stearic acids in whole fats.

	(M)	SC	SB	SA	N
Myristic-palmitic	52·0	54·2	62·5	67·7	73·2
Stearic	48·0	45·8	37·5	32·3	26·8

Relative proportions of myristic-palmitic and stearic acids in the non-fully-saturated glycerides.

	(M)	SC	SB	SA	N
Myristic-palmitic	46·6	47·6	63·6	68·0	77·1
Stearic	53·4	52·4	36·4	32·0	22·9

The widely varying proportions in the whole fat are reduced to something approaching constancy in the fully-saturated glycerides and correspondingly accentuated in the non-fully-saturated glycerides.

It may also be remarked that the "association ratio" with respect to stearic acid in the fully-saturated glycerides is usually approximately 1·5 mols. of palmitic (with myristic) acid per mol. of stearic acid, a value which, curiously, is the same as that in which acids present in major proportions are usually found linked with acids present in minor proportions in vegetable seed fats; it is not likely, however, that this connotes any great similarity in mode of assemblage, since the presence of definite though small amounts of tristearin and tripalmitin indicates that the distribution of the fatty acids is less even in the fully-saturated parts of animal fats than in seed fats.

The foregoing results lead to the conclusion that the body fats of the ox (and probably also the sheep) are made up of a mixture of fully-saturated components, tending towards constancy in composition, with non-fully-saturated glycerides in which the amounts of stearic and oleic acids may vary considerably, but that the sum of these two acid components is nevertheless more or less constant and typical. These general tendencies must be intimately connected with the metabolic processes which are involved in the deposition of storage fat. It is difficult to draw conclusions as to the nature of such processes from an examination of their end-products, but the following points arising out of the above data may be noticed.

1. The consistency or melting-point of a body fat is generally agreed to be more or less adapted to the environment of the animal, and it has usually

been defined hitherto as being determined by the proportion of liquid or unsaturated fatty acids which are present. The present data suggest further that variation in consistency is obtained by fluctuation in the relative amounts of stearic and oleic acids, subject to the condition that the united proportion of these acids remains approximately constant. Also, the melting-point of the fat is determined by the presence of varying amounts of relatively high-melting fully-saturated glycerides of approximately constant composition accompanied by inversely varying proportions of glycerides containing oleic acid, which will tend to be lower or higher in melting-point according to the presence of less or more stearic acid in the approximately constant amount of total C_{18} acids.

2. The manner in which the fatty acids are assembled into body fats constituted in conformity with these generalisations forms an interesting subject for discussion. The well-established fact that animal fats are assembled in an essentially different manner from vegetable seed fats (although not very differently from fruit-flesh fats and, therefore, possibly, from leaf fats) reinforces the accepted view that ingested fats are hydrolysed and resynthesised into glycerides in the animal. Thus, assuming that a given mixture of palmitic, stearic, oleic and linoleic acids is available for laying down as depot fat, it appears that the glyceride synthesis operates in such a way that there is an approximately constant molar proportion of stearic and oleic (total C_{18}) acids in the product; at the same time, a certain amount of glycerides containing only stearic and palmitic (with myristic) acids is laid down and this portion always contains about 37–40 % of stearic residues. The approximately constant balance between the C_{18} and the C_{16} (*plus* C_{14}) acids in the whole fat, despite varying stearic acid content in the latter, is then maintained by variation in the amount of unsaturated acid in the non-fully-saturated portions, and also in the relative amounts of fully-saturated and non-fully-saturated glycerides.

So far as is known at present, the components of vegetable fats normally ingested by cattle are relatively small amounts of palmitic acid with smaller but variable amounts of stearic acid accompanied by considerable proportions of oleic acid and, usually, still more linoleic acid[1]. It is conceivable that from such a mixture of fatty acids glycerides may be laid down in conformity with the generalisations concerning their structure at which we have arrived above, the available palmitic and stearic acids being combined so as to give the more or less constant proportions of palmitic acid in the whole fats by inclusion of the requisite amount of oleic and linoleic acids to balance the stearic acid which is also utilised. In the tallows the amount of oleic and linoleic acids is definitely lower than in the plant oils which we visualise as being the main source of the fatty acids assimilated by the animal, and there must remain an excess of these after the body fat has been synthesised, and this excess

[1] The recent work of Barber [1929] has shown that mixtures of these four acids are also produced from carbohydrate material by ferment action.

may well be disposed of by oxidation; some such method of elimination of the excess of linoleic acid appears to be necessary, moreover, to account for the characteristic and relatively small ratio of linoleic to oleic acid in the tallows, and also for the quantitatively similar ratio in the milk fats.

It should be emphasised, also, that the relative proportion of stearic to palmitic acid in the whole tallows is as a rule much higher than that in any fatty component likely to be present in the diet of cattle, and that therefore we are faced either with the possibility that palmitic acid is destroyed, or with the more likely alternative that stearic acid is synthesised, either from corresponding unsaturated acids or from non-fatty material, in the animal organism. In either of these latter alternatives, it appears that a process of "saturation" rather than "desaturation" predominates on the whole during the elaboration of tallows, and we consider, with Leathes and Raper [1925], that "desaturation" appears unlikely as a process which operates in the synthesis of unsaturated acids previous to their storage as neutral fat.

We hope to obtain further insight into the problems discussed above from a parallel study, at present in progress, of the body-fats of the pig, in which we are investigating the fats from a single animal fed on a controlled diet.

Summary.

Four beef tallows from various North and South American sources have been investigated with reference to their content of fully-saturated glycerides and the component fatty acids of the latter and of the whole fats.

In the whole fats the united molar percentage of stearic, oleic and linoleic acids tends towards one of two definite figures (*ca.* 62 or *ca.* 69 mols. %) in spite of wide variations in the amount of stearic, and correspondingly of oleic (with linoleic), acid. This relation is also shown to subsist in a number of other tallows for which previous analyses are available.

The proportion of fully-saturated glycerides present varies according to the relative total amounts of saturated and unsaturated acid in the whole fats, but the composition of the acids present therein tends to much the same figure in all cases, namely, about 60 % of palmitic (with myristic) acid and about 40 % of stearic acid.

These regularities in structure, which are strikingly similar to those recently observed in a series of butter fats, are discussed in regard to their possible implications concerning the processes involved in the elaboration of storage fat in the animal.

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CXXXI. DETERMINATION OF INORGANIC SULPHATE IN HUMAN BLOOD-PLASMA BY MICRO-TITRATION.

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(*Received June 30th, 1931.*)

Observations on the inorganic sulphate content of human blood and other body fluids are relatively infrequent, partly owing to the great difficulties encountered in estimating the small concentrations of inorganic sulphate present therein, but also because the sulphate ion has been regarded as pharmacologically inert and as therefore of little importance in the elucidation of physiological and pathological problems. That it is pharmacologically inert is probably true. The intravenous injection of sodium sulphate into human beings in amounts up to 3 or 4 g. certainly has no detectable effects save an increase in the amount of sulphate excreted in the urine[2], and Sollman [1926] states that much larger amounts may be given without demonstrable effects.

There are however certain problems in the solution of which a knowledge of the blood sulphate would be of considerable value. Peters and his associates [1929] have called attention to the probable rôle of sulphate in accounting in part for the "undetermined acid" combining with base in cases of nephritis with nitrogen retention and have made a plea for further investigation of its behaviour in the organism.

The suggestion that sulphate, being apparently merely filtered off by the renal glomeruli and not again re-absorbed in the tubules, could be used as a measure of the volume of glomerular filtrate [White, 1923; Rehberg, 1926; Sager, 1930; Poulsson, 1930], has greatly increased the need for a satisfactory method of estimation of inorganic sulphate in small quantities of blood-plasma or serum. Rehberg considered the estimation too difficult in plasma. White and also Sager, simplified the problem by artificially increasing the blood sulphate concentration to more easily estimated levels, whilst Poulsson contented himself with a rough estimate of the probable concentration in the blood during his experiments.

Numerous methods for plasma inorganic sulphate estimation have from time to time been described. The more recent of these may be classified as follows.

(*a*) *Gravimetric*, Meyer-Bisch [1924], Pohorecka-Lelesz [1927], Loeb and Benedict [1927].

[1] Beit Memorial Research Fellow.

[2] Personal observation, unpublished.

(*b*) *Nephelometric*, Denis [1921], Denis and Reed [1926], Chatron [1931].

(*c*) *Benzidine, by titration*, White [1923], Sager [1930], Pohorecka-Lelesz [1927], Chatron [1931], Lesure and Dunez [1929].

(*d*) *Benzidine, colorimetric*, Yoshimatsu [1926], Wakefield [1929], Hubbard [1930].

(*e*) *Sulphide formation*, Lorant [1929].

(*f*) *Miscellaneous*, Lang [1929].

Of these different methods, the gravimetric requires either a large volume of blood or very accurate weighing on a micro-balance. The nephelometric method suffers from the fact that the turbidity of the suspension of barium sulphate is very dependent on changes in the p_H and composition of the blood-filtrate. The titration of benzidine sulphate has, so far as the writer is aware, only been applied to blood in which sulphate concentration has been artificially raised, or in which it is already higher than in human blood [Pohorecka-Lelesz, 1927].

The colorimetric benzidine processes require great care in their manipulation if colour is to remain proportional to sulphate content. The colours obtained, moreover, being weak yellows and browns, are difficult to compare accurately, at least for some workers.

Reduction to a sulphide is a somewhat laborious procedure and appears to include ethereal and other sulphates in the analysis. Finally, the method of Lang, whilst giving excellent pink colours, has not, in the hands of the writer at least, given constant results with such low concentrations of sulphate as are encountered in normal human plasma.

Numerous attempts have been made by the writer to improve on one or other of these methods. A modification of the Denis and Reed nephelometric procedure and a micro-titration method based on Hubbard's technique [1930] will be described.

Nephelometric method.

Denis's claim for an accuracy of 5 % for her modified method [1926] has been fully confirmed for calf's and sheep's serum in both of which the actual inorganic sulphate concentration is five or six times as high as in human serum. Table I shows the results obtained.

Table I. *Comparison of gravimetric and Denis nephelometric methods.*

Serum used	Gravimetric S mg./100 cc.	Nephelometric S mg./100 cc.	Error (%)
Calf's serum	5·29	5·36	1
,, ,,	7·16	6·94	3
Sheep's serum	3·59	3·73	4
,, ,,	3·51	3·73	6
Calf's serum	4·77	4·58	4
,, ,, (with added S)	5·25	5·45	4
,, ,, (diluted 1 : 1)	2·38	2·40	1
Sheep's serum	3·12	3·06	2
,, ,, (with added S)	3·74	3·71	1

When however the same method was applied to human plasma or serum, the results were far less satisfactory. Denis herself stated that, if from a blood-filtrate no precipitate is obtained with barium chloride, it is necessary before concluding that no sulphate is present to determine the p_H of the solution, the optimum value for which she gives as 3·0 to 3·8. The method as described by Denis, however, takes no account of the p_H of the final filtrate, but assumes that in all ordinary cases this will be such as to give a reliable nephelometric reading, if the reagent quantities given are adhered to.

It was felt that herein lay a possible source of the discrepant results, since, if an unfavourable p_H may completely prevent the precipitation of barium sulphate, then one somewhat less unfavourable may evidently seriously affect the resultant turbidity without necessarily arousing suspicion. This was found actually to be the case, and a p_H range of from 1·8 to 2·4 was found to yield maximum and fairly constant turbidities. This is rather more acid than the p_H recommended by Denis and slightly more so than that suggested by Chatron, but the small differences may be due to variations in the gelatin stabilisers employed.

It was, however, found that even with a constant p_H, the total concentration of trichloroacetic acid present had a serious effect on the turbidity produced. This is well shown in the following example in which a solution with a sulphate content corresponding to the filtrate from a plasma with 0·65 mg. S per 100 cc. was employed.

Cc. 20 % trichloroacetic acid present in 15 cc. of filtrate	0·1	0·25	0·5	0·75	1·0	1·5
Final p_H to thymol blue	2·5	2·4	2·4	2·3	2·4	2·2
Percentage error in estimation	6	3	6	20	28	43

The presence of small quantities of trichloroacetic acid in the solution did not materially influence the result. The excess can be conveniently removed by shaking the protein-free blood-filtrate with two volumes of ether, if allowance be made for the resultant change in volume of the water phase (about 7 %). Much more consistent results are then obtained, as is shown in the following example, in which the same concentration of sulphate as above was used.

Cc. 20 % trichloroacetic acid present in 15 cc. of filtrate	0·25	1·5	1·5	1·5
Final p_H to thymol blue	2·0	2·0	2·0	2·0
			Extracted with ether	
Percentage error in estimation	1	32	1	3·5

The same effect was observed with other low concentrations of sulphate.

It is evident therefore that in order that the nephelometric method should yield even approximately constant results with human plasma, (*a*) the p_H of the solution must be controlled in every case and brought to the optimum value; (*b*) the quantity of trichloroacetic acid present in the filtrate must be small.

The presence of ether in the extracted solution appeared to have little effect on the nephelometric readings, but it is desirable to treat the standard sulphate solutions in a similar manner.

Denis's method modified in this manner was employed for some time by the writer with fairly satisfactory results but was finally abandoned in favour of the micro-titration benzidine technique. As this has proved itself to be in every way preferable to the modified nephelometric method, details of technique of the latter will not be given.

Both procedures give figures varying from 0·5 to 1·5 mg./100 cc. of S as inorganic sulphate for normal human plasma and thus agree with the now generally accepted values.

Micro-titration benzidine method.

This is a modification of the method advocated by Hubbard [1930] and indeed follows his procedure in detail as far as the production of the washed benzidine sulphate precipitate. This Hubbard estimated colorimetrically, but estimation by direct titration has been found more reliable for low sulphate concentrations. For convenience, Hubbard's procedure is described here. For further details the original paper should be consulted.

Reagents.

(*a*) 1 % benzidine in good acetone.
(*b*) 20 % sulphate-free trichloroacetic acid in water.
(*c*) Good grade acetone.
(*d*) *N*/50 CO_2-free sodium hydroxide.

Method.

To 2 to 5 cc. of serum or citrated plasma in a centrifuge tube is added an exactly equal volume of 20 % trichloroacetic acid, and the whole is thoroughly mixed with a thin glass rod. The precipitated proteins are centrifuged, leaving a clear supernatant fluid which usually requires no further filtration.

In a 10 cc. Pyrex glass centrifuge tube with sharply pointed tip are placed 5 cc. of the 1 % benzidine in acetone solution, and to this are added exactly 2 cc. of the protein-free blood-centrifugate. The tube is stoppered and kept in ice-water for an hour, or until flocculation of the precipitate begins to occur. It is centrifuged and drained for 5 minutes, the tube being inverted in a small beaker in the bottom of which is a pad of filter-paper moistened with acetone. This prevents evaporation of the acetone before drainage is complete. 10 cc. of fresh acetone are added, the precipitate is broken up with a fine glass rod and centrifuged; the tube is again drained on the pad for 5 minutes.

Titration. For this purpose the micro-burette described by Rehberg [1925] has been found eminently suitable. It should be filled with *N*/50 CO_2-free sodium hydroxide. The titration is carried out in the Pyrex centrifuge tube itself, this being surrounded by a steam-jacket. The apparatus recommended is shown in Fig. 1.

The jacket is readily made from ordinary glass. Steam enters by the side-tube from a suitable flask, and escapes with the condensed water into a

receiver placed below the bottom tube. The upper opening carries a rubber stopper bored with a hole into which the centrifuge tube fits.

Mixing of the solution during titration is effected by bubbling through it a slow stream of CO_2-free air, most conveniently obtained from two large bottles connected by a syphon and containing weak sodium hydroxide solution. The rate of bubbling is controlled by a screw-clip.

To the tube containing the washed and drained benzidine sulphate precipitate is added 0·5 cc. of distilled water. Phenolphthalein is added by dipping the tip of a fine glass rod into the indicator solution and inserting it into the centrifuge tube, where it may be allowed to remain, as it is useful for breaking up the precipitate during the titration. The centrifuge tube with its fine glass stirring rod is inserted into the steam-jacket, and the whole arranged so that the tip of the microburette just dips under the surface of the liquid in the tube. The tube for bubbling CO_2-free air is next placed in position and a gentle stream commenced.

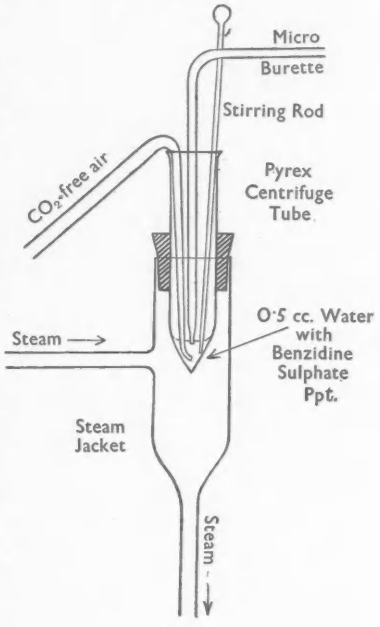

Fig. 1.

It is advisable to carry the titration as far as possible before the steam is turned on. When the pink colour due to the added alkali tends to become persistent, the steam is turned on and the titration completed as rapidly as possible at 100°. No difficulty is encountered in detecting the first appearance of a permanent pink if good lighting be arranged. Before removing the tube its sides are well washed down by turning on the air supply fully for a moment, and further alkali is added if necessary. The centrifuge tube and jacket are then immediately removed from the tip of the burette. Some haste is desirable during these later stages in order to minimise the heating of the fluid in the burette during the titration. After the tube is withdrawn cooling leads to a slight retraction of fluid from the tip of the burette. The meniscus must be brought once again to the tip by turning the control screw, before the final reading of the burette is made.

A blank titration is necessary in order to determine the amount of alkali required to produce a definite pink colour in 0·5 cc. of distilled water at 100°. This usually lies between 3·0 and 4·5 mm.^{3} of $N/50$ sodium hydroxide, and the blank must be subtracted from the titration figure.

This procedure has given more reliable results than any other tried by the writer. The figures in Table II indicate the degree of accuracy obtained.

All the determinations were performed on samples of sheep's serum which had been dialysed for 14 days against pure 0·9 % sodium chloride. It is interesting that, in the second specimen of serum, sulphate in a concentration of 0·06 mg. S per 100 cc. could be definitely detected, and produced a distinctly visible deposit of benzidine sulphate.

Table II.

Number	Added S mg./100 cc.	Theoretical S mg./100 cc.	Added citrate mg./100 cc.	Added P mg./100 cc.	mm^3 $N/50$ sodium hydroxide used (corrected)	Estimated S mg./100 cc.	(%) recovery
1	0·83	0·83	—	—	26·1	0·836	100
2	1·15	1·15	—	—	38·2	1·22	108
3	1·15	1·15	—	—	35·1	1·12	98
4	1·43	1·43	—	—	43·3	1·38	96
5	1·43	1·43	—	—	43·2	1·38	96
6	1·37	1·37	200	—	41·4	1·32	96
7	1·37	1·37	200	—	41·3	1·32	96
8	1·67	1·67	—	—	52·5	1·68	100·6
9	1·67	1·67	—	—	52·0	1·665	100
10	1·62	1·62	200	—	51·6	1·65	102
11	1·62	1·62	200	—	49·0	1·565	96·6
12	2·14	2·14	—	—	69·0	2·20	103
13	2·14	2·14	—	—	67·3	2·15	100·5
14	1·67	1·67	—	3·3	53·4	1·71	102·4
15	1·67	1·67	—	3·3	52·9	1·69	101·2
16	1·36	1·36	—	6·5	41·6	1·33	97·8
17	1·36	1·36	—	6·5	43·0	1·375	100·7
Fresh dialysed serum estimated to contain 0·06 mg. S per 100 cc.							
18	2·31	2·37	—	—	76·0	2·44	103
19	2·31	2·37	—	—	77·0	2·46	104
20	0·91	0·97	—	10·0	30·4	0·97	100
21	0·91	0·97	—	10·0	32·1	1·02	105
22	1·67	1·73	—	10·0	54·5	1·74	100·6
23	1·67	1·73	—	10·0	55·2	1·76	102
24	0·55	0·615	—	12·3	20·1	0·64	104
25	0·55	0·615	—	12·3	18·6	0·595	96·7
26	2·00	2·06	—	22·0	74·0	2·37	115
27	2·00	2·06	—	22·0	74·0	2·37	115
28	0·45	0·515	—	10·0	16·4	0·525	102
29	0·45	0·515	—	10·0	17·2	0·55	107
Third sample of dialysed serum free of sulphate.							
30	0·91	0·91	900	5·0	28·2	0·905	99·5
31	0·91	0·91	900	5·0	27·9	0·896	98·6
32	2·86	2·86	900	5·0	101·0	3·23	112
33	2·86	2·86	900	5·0	97·0	3·11	108
34	3·33	3·33	900	5·0	111·0	3·56	107
35	3·33	3·33	900	5·0	114·0	3·64	109
36	3·85	3·85	900	5·0	129·0	4·14	107·5
37	3·85	3·85	900	5·0	130·0	4·16	108

The figures show that even a large excess of citrate has no appreciable effect on the estimation.

Concentrations of phosphate up to 12 mg. P per 100 cc. of original plasma have no effect on the results, and the figures show a satisfactory agreement with the theoretical up to about 2·5 mg. S per 100 cc. but tend to be a little too

high when the sulphate concentration exceeds that value, and much phosphate is present.

SUMMARY.

Some errors in the nephelometric method of estimating inorganic sulphate in small amounts of human plasma or serum are considered, and means of overcoming them are suggested.

Details of a modified benzidine technique are given in which the more usual colorimetric estimation is replaced by micro-titration with sodium hydroxide. With the filtrate from 1 cc. of human serum or plasma this method gives results within 5 % of the theoretical.

The author wishes to express his thanks to Prof. R. A. Peters for many helpful suggestions, and to Dr J. G. Priestley for the loan of Rehberg microburettes.

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CXXXII. THE IODINE METHOD FOR THE DETERMINATION OF GLUTATHIONE.

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The usual and more practical method for the estimation of glutathione in the tissue extracts is considered to be Tunnicliffe's iodine method [1925]. The end-point of the reaction is determined by the use of either starch or sodium nitroprusside as indicator. In both cases there are some difficulties in determining the true end-point. With the starch as internal indicator the end-point is very uncertain; the initial blue colour, once attained, persists for an interval of time varying from 5 to 30 seconds with the different tissue extracts, and this time of persistence increases with the amount of iodine added after reaching the initial blue colour. With very pure samples of glutathione the end-point is much more easy to determine with precision. It is probable that this trouble is introduced by the presence in the extracts of foreign substances which react with the iodine with different speeds so that the final figures will always be larger and will not represent the true amount of the glutathione present in the tissue extracts.

The sodium nitroprusside end-point is more accurate and the difficulty in using it consists only in appreciating the point of disappearance of the red colour.

All authors have remarked on the discrepancy between the figures obtained, using starch or nitroprusside as indicator.

Voegtlin and Thompson [1926] believe that the starch is not specific enough as indicator. Kennaway and Hieger [1927], in their experiments, found large differences (from 40 to 50 %) between starch and nitroprusside end-points. Bierich and Kalle [1928] came to the same conclusions. Furthermore, Mason [1930] assumes that the method is not only subject to the error introduced by these modes of determining the end-point, but even the iodine estimation itself is not trustworthy for some tissue extracts, for example: liver, kidney and yeast. He compared the iodine method with a colorimetric one, based on the oxidation of the .SH group in glutathione with potassium ferricyanide. Mason gives a table (IV) showing results which are very suggestive, but it is difficult to understand the wide variation in the individual figures in his estimation of glutathione from yeast, using nitroprusside as external indicator. In the present experiments, using the same sample of yeast extract, the numerous estimations performed with nitroprusside as indicator, have always

given very consistent results, provided that the nitroprusside test is performed under very accurate and standardised conditions, as suggested for instance by Kennaway and Hieger [1927]

Considering the difficulties in obtaining concordance in the results of glutathione estimation from tissue extracts with the starch-iodine method, Perlzweig and Delrue [1927] proposed a modification of Tunnicliffe's method consisting in addition of an excess of 25 % potassium iodide (2 cc. for each sample ready for estimation). According to their view the potassium iodide stabilises the end-point; this is also the experience of the present author. However, the potassium iodide introduces another error if we do not take into account the acidity of the sample.

The present experiments are therefore a comparative study of the estimation of glutathione in pure solutions of this substance and in tissue extracts using one of the following indicators.

(1) Starch in ordinary acid extracts.
(2) Starch, acid solution and 2 cc. of 25 % potassium iodide.
(3) Starch *plus* potassium iodide in sample neutral to litmus.
(4) Nitroprusside.

The results are shown in the Tables.

Table I. *Glutathione estimations in various organs.*

For each estimation 10 cc. of extract.

	cc. *N*/100 iodine			
		2 cc. of 25 % KI		
	No KI; acid	Acid	Neutral to litmus*	Nitro-prusside end-point
Sheep brain†; approx. 50 g. tissue for 150 cc. extract	1·50	0·56	0·86	0·80
	1·56	0·54	0·84	0·80
	1·54	0·54	0·84	0·80
	1·54	0·54	—	0·80
Beef; approx. 50 g. tissue for 150 cc. extract	0·7	0·36	0·54	0·50
	0·7	0·32	0·56	0·50
	0·7	0·36	0·50	0·50
	0·7	0·36	0·54	—
Yeast; approx. 50 g. for 250 cc. extract	1·44	1·16	1·40	1·44
	1·44	1·18	1·42	1·46
	1·44	1·14	1·44	1·44
	1·44	1·14	1·42	1·46
	1·44	1·16	—	—
Liver; approx. 50 g. tissue for 250 cc. extract	2·46	0·86	1·1	1·2
	2·42	0·82	1·1	1·2
	2·46	0·82	1·2	1·2
	2·40	0·82	1·1	1·2
Glutathione 0·225 %. Very pure sample. For each estimation 1 cc. in 10 cc. of 10 % trichloroacetic acid	0·92	0·76	0·88	0·9
	0·92	0·76	0·9	0·9
	0·90	0·78	0·86	0·9
	0·90	0·78	0·88	—

* The neutralisation was effected by addition of 2*N* NaOH.

† These figures do not represent the real amount of the glutathione present in tissues because the latter have been washed only two or three times.

To avoid any misunderstanding it is necessary to consider some of the major points of the method.

The tissue extracts have been made with pure (Merck) trichloroacetic acid (10 %). The filtrate was clear. The potassium iodide solution was prepared by dissolving a pure sample in freshly boiled and cooled water. Fresh solutions have always been used and were placed in the dark immediately after preparation. The nitroprusside test was performed according to the suggestions given by Kennaway and Hieger [1927].

Table II. *The effect of varying concentrations of KI in acid and neutral solutions.*

	cc. of 25 % KI	cc. N/100 iodine: Acid	cc. N/100 iodine: Neutral
Liver	0·0	1·84 and 1·84	1·1 and 1·1
	0·1	1·3 „ 1·34	1·06 „ 1·1
	0·2	1·08 „ 1·08	—
	0·5	0·92 „ 0·94	—
	1·0	0·82 „ 0·82	1·1 „ 1·08
	2·0	0·68 „ 0·68	—
	3·0	—	1·06 „ 1·08
Glutathione 0·225 %	0·0	0·90	0·88
	0·1	0·82	0·86
	0·2	0·82	0·88
	0·5	0·82	0·88
	1·0	0·80	0·86
	2·0	0·76	0·88
	3·0	0·70	0·84 and 0·86

Table III. *The effect of* p_H *on the glutathione estimation in the presence of excess of potassium iodide.*

Glutathione 0·25 % (for each estimation 1 cc. glutathione solution *plus* 10 cc. phosphate mixture *plus* 2 cc. 25 % KI).

						Strongly acid (control)	
p_H	5·9	6·46	6·8	6·9	7·16	KI	No KI
cc. iodine N/100	0·80	0·84	0·88	0·94	1·00	0·8	1·0
	0·82	0·84	0·88	0·94	1·00	0·8	1·0
	0·80	0·84	—	0·92	—	0·8	—

We notice that the depressant action of the potassium iodide is present even with samples of very pure glutathione (Table I).

Table IV. *Sheep brain.*

70 g. for 250 cc. extract; comparative estimation of the glutathione in acid or neutral solution, with or without KI.

Acid			Neutral	
No KI	2 cc. of 25 % KI	Nitroprusside end-point	No KI	2 cc. of 25 % KI
1·34	0·50	0·74	1·22	0·76
1·38	0·52	0·78	1·16	0·8
1·42	0·50	0·76	1·2	0·78
1·38	—	0·74	1·2	—
—	—	—	1·2	—

Discussion.

This error introduced by the presence of potassium iodide was noticed by Okuda [1925] in his experiments on the estimation of cysteine. He believed that the discrepancies might be explained by assuming different chemical reactions between cysteine and iodide under the different conditions of estimation, but he did not prove this point experimentally. However, he correlated the difference in the reaction with the temperature, acidity and presence of potassium iodide. To avoid the effect of these different factors which interfere with the results, he proposed for the cysteine estimation specially standardised conditions.

If we consider now the figures from the present paper, we notice the following very suggestive facts.

In the estimation of pure samples of glutathione there is no difference between starch-iodide (no KI) and nitroprusside end-points.

The same statement holds for the yeast samples. The same comparison for beef, brain and liver extracts gives respectively the average differences 29 %, 47 %, and 50 %. Do these differences between starch and nitroprusside end-points represent some foreign substances interfering with the estimation of the glutathione? Probably, but if so, why are there no differences between these figures when the estimation with starch as indicator is made in neutral solution?

Indeed we notice from our Tables that, if the nitroprusside end-point in the estimation of .SH groups is the most specific one, the iodine-starch-KI method may be very accurate, provided we take into account some special condition with regard to the acidity of the sample.

In acid solution the use of excess KI produces lower figures than the nitroprusside. Near, or just at the neutral point of the solution, the figures obtained with excess potassium iodide are concordant with the nitroprusside figures (Tables I and II).

If the estimation is made at the neutral point but without adding potassium iodide in excess, the figures obtained are higher than those indicated by nitroprusside. Table IV shows this comparative experiment. Therefore the KI seems to be necessary for a good estimation.

Table III is a good demonstration of the relations between the p_H and the estimation of pure glutathione when the KI is present in excess.

The depressant action of KI in acid solution depends also upon its concentration (see Table II), but by neutralising the sample this effect of concentration is eliminated. What is the explanation of this fact?

First we must remember that in the ordinary estimation of sodium thiosulphate with iodine, the addition of KI in acid solution in no way affects the end-point, using starch as indicator. A fundamental difference between the thiosulphate and the glutathione estimations seems to be that in the former there is no liberation of HI, whereas HI is liberated in the latter. The trouble may thus be introduced by the formation of the HI which in the presence of

KI reaches some equilibrium with the liberation of free iodine. That the amount of free iodine is correlated with the p_H of the sample is suggested by our experimental results (see Table II).

We can consider a second explanation. In acid solution, in the presence of certain compounds contained in the tissue extracts, the KI could liberate iodine, and the free iodine would then react with the .SH groups to some extent before the titration was commenced. This view is not supported by the following experimental fact. If we leave some glutathione or extract in the presence of 2 cc. of 25 % KI, estimating then the glutathione at different time intervals, we find no difference between the figures.

The most probable explanation is the physico-chemical explanation of equilibrium between KI-HI-I, this equilibrium being correlated with the p_H of the sample. Further investigation will perhaps indicate how the equilibrium is to be formulated.

Conclusions.

A comparative study has been made of glutathione estimation in tissue extracts, either with starch or nitroprusside as indicators.

In comparison with the nitroprusside end-point the starch in acid samples and without KI gives very high figures. In the presence of excess of KI on the contrary, lower results are obtained.

The figures obtained in the estimation of glutathione in pure solution and in tissue extracts, using excess of potassium iodide and making the sample neutral to litmus, are concordant with the figures obtained, using the nitroprusside end-point.

The author proposes the above modification of Tunnicliffe's method which is very accurate when compared with the methods employing nitroprusside as an indicator.

I wish to express my sincere gratitude to Prof. R. A. Peters for his criticism, and to thank Dr Dixon for some samples of pure glutathione.

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CXXXIII. THE FATE OF CAROTENE AFTER ABSORPTION IN THE ANIMAL ORGANISM.

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THERE is now ample evidence that administration of carotene to animals fed on a vitamin A-deficient diet is followed by a return to normal health and the appearance in the liver of a substance showing the properties of vitamin A [Moore, 1929, 1930, 1931; Drummond, Ahmad and Morton, 1930; Capper, 1930; Capper, McKibbin and Prentice, 1931]. In investigating the quantity of carotene that must be given to restore a normal rate of growth in rats considerable variations in the response of the animals were encountered. The irregularities were such as to suggest that they arose from imperfect absorption of the pigment from the gut. The examination of the excreta showed that there were adequate grounds for this view, in that a considerable proportion of the pigment could be recovered from the faeces. At the time this observation was first made the basal diet being employed was almost devoid of fat and the carotene was being administered in the form of a solution in ethyl laurate. This suggested that the absence of fat might be the cause of the poor absorption of the pigment, and a series of experiments was planned to test this view.

Three types of basal diet were used. They were compounded as follows:

1. Fat-free diet

Purified caseinogen[1]	22
Pure glucose	73
Salt mixture	5

2. Low fat diet

"Light-white casein" (B.D.H.)	20
Rice starch	71
Salt mixture	5

[1] The caseinogen was purified by two precipitations with acetic acid and thorough washing after each precipitation. After pressing out the excess of water, the granular mass was dried by treatment with absolute alcohol, then with ether and finally in a hot air-oven at 90° for a few minutes. The dried product was ground to a fine powder and extracted with ether in a Soxhlet extractor for 72 hours.

3. Fat-containing diet

"Light-white casein"	20
Rice starch	61
Hardened cottonseed oil ...	10
Salt mixture	5

All the animals received in addition a daily supplement of 0·003 mg. of irradiated ergosterol dissolved in liquid paraffin and a preparation of an alcoholic extract equivalent to 0·5 g. of dried brewer's yeast. When the animals were showing signs of vitamin A deficiency, known amounts of carotene were administered, either in the form of a solution of the pure pigment in ethyl laurate or as palm oil. The excretion of the pigment by the alimentary tract was estimated colorimetrically.

The results are given in Table I.

Table I.

Experiment	Diet	Carotene administered	% carotene recovered from the faeces
1	(1) Fat-free	0·5 mg. in ethyl laurate	84·9
2	,,	,, ,,	86·5
3	,,	,, ,,	91·8
4	,,	0·1 mg. as palm oil (60 mg. of the oil)	40·9
5	(2) Low fat	0·5 mg. in ethyl laurate	91·3
6	,,	,, ,,	85·5
7	,,	0·1 mg. as palm oil (60 mg. of the oil)	38·1
8	,,	,, ,,	38·0
9	,,	,, ,,	38·0
10	,,	,, ,,	40·0
11	,,	0·1 mg. in ethyl laurate	93·7
12	(3) 10 % fat	,, ,,	11·7
13	,,	,, ,,	11·7

On the assumption that the yellow colour of the fats extracted from the faeces was due solely to carotene, these results illustrate in a striking manner how the absorption of the pigment from the intestinal tract may be influenced by the composition of the diet. Moore [1931] has recently reported that the fat extracted from the excreta of rats fed for a long period on a diet containing 15 % of red palm oil contained unchanged carotene in a concentration as high as twelve times that of the ingested oil.

It was, however, necessary to ascertain that the extracted pigment was indeed carotene. For this purpose a more detailed colorimetric and spectroscopic examination was made of the fats extracted from the faeces and the contents of the caecum of rats fed on a fat-free diet supplemented by a high daily dose of 0·5 mg. of carotene dissolved in ethyl laurate.

The examination of the faecal fats showed that the yellow colour was, within experimental error, entirely due to carotene. In the case of the fats extracted from the contents of the caecum, however, results of considerable interest were obtained. Measurement of the yellow colour and of the blue

colour produced with antimony trichloride showed that carotene was not the only chromogenic substance present. The carotene content measured spectroscopically was 1·4 %, while on the basis of the intensity of the 590 $m\mu$ band produced with antimony trichloride the percentage calculated was 2·7 %. Carotene accounted, therefore, for only some 50 % of the blue colour. Spectroscopic examination of the fat in 1 % solution in alcohol in 2 mm. thickness showed a well-defined band at 338 $m\mu$ (Curve *A*, Fig. 1). By referring to the

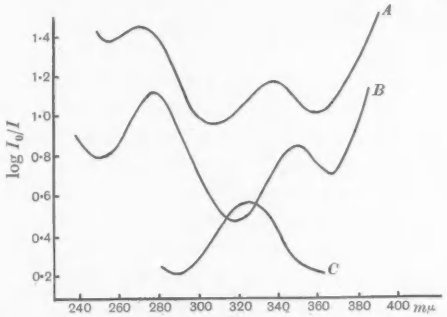

Fig. 1. Spectroscopic examination of the caecum fat. Curve *A*. Caecum fat 1 % solution in alcohol 2 mm. thickness. Curve *B*. Carotene content of the caecum fat calculated theoretically. Curve *C*. By subtracting *B* from *A*; a typical vitamin A curve; maximum absorption about 325 $m\mu$.

absorption curve of pure carotene (Curve *B*), it can be concluded that the fat was exhibiting a band at about 325 $m\mu$ of a similar character to that associated with vitamin A (Curve *C*).

If the substance responsible for this band at 325 $m\mu$ was indeed vitamin A, the question arose as to its origin. The alternative explanations were that it was formed in the intestinal tract, presumably by bacterial action, or that it had been formed in the tissues and excreted into the gut by way of the bile. In order to test the first possibility a series of experiments was made on the influence of intestinal bacteria on carotene under different conditions. Colloidal solutions of carotene were prepared, usually by pouring alcoholic or ethereal solutions of the pigment into water, gum saline or broth, shaking the mixtures well and removing the solvents carefully. The addition of small amounts of cholesterol greatly facilitates the preparation of such suspensions and renders them more stable. Such carotene preparations mixed with peptone broth media were inoculated with material from the caeca of the rats that had been kept for some time on a vitamin A-deficient diet, and the cultures were incubated either aerobically or anaerobically at 37° for periods ranging from 1 to 11 days. After incubation the fat-soluble material was extracted by ether and the dried extract was examined for its yellow colour and for its power to give the blue colour with antimony trichloride. A very large number of cultures of this type were made. The results were very inconsistent when judged by the yellow-blue

ratio, which, as Moore [1930] has pointed out, is about 11 for pure carotene. In general the aerobic cultures showed a reduction in both yellow and blue colours pointing to conversion of carotene into products not giving the antimony trichloride reaction. The anaerobic cultures gave very irregular results. In some cases the carotene was apparently unaffected by the bacterial growth, in others some destruction similar to that in the aerobic tubes occurred, whilst in two cases very definite evidence of the conversion of carotene into a substance giving a much more intense reaction with $SbCl_3$ was obtained. The yellow/blue ratio, which for other cultures showed values ranging between 4 and 11, was 1–1·3.

In one case the change was very striking in that the extracted material had lost most of its yellow colour but gave a much more powerful reaction with $SbCl_3$ than the original pigment. The product appeared to be very unstable, for after some hours the reaction with $SbCl_3$ was much less intense, and by the time the sample was examined spectroscopically by Dr Morton the chromogenic property had disappeared. In spite of many attempts, it has not been found possible to reproduce the conditions that gave this interesting result, and it is uncertain, therefore, whether a formation of vitamin A from carotene was brought about by anaerobic bacteria in these two isolated experiments.

The failure of the large proportion of these experiments to demonstrate the production of vitamin A from carotene by intestinal bacteria diverted attention to the possibility that the conversion normally takes place after absorption.

If it be assumed that the change takes place in the tissues, it must be admitted that there is a large amount of evidence to support the view that the liver is the organ chiefly concerned. Sherman and Boynton [1925] and Kerppola [1930] have shown that the liver of the rat contains much more vitamin A than its other organs, whilst the recent work of Moore [1931] and Capper, McKibbin and Prentice [1931] proves the remarkable concentration of the vitamin in the liver that may follow the administration of carotene. Of no less significance are the observations of Channon [1926] and of Channon and Collison [1928, 1929] on the retention in the liver of the greater part of the unsaponifiable substances (*e.g.* cetyl alcohol, liquid paraffin) absorbed from the intestinal tract.

It appeared to be of importance, therefore, to obtain more precise information regarding the rôle of the liver in the formation of vitamin A from carotene.

In the first place the effect of incubating pulped liver tissues with colloidal suspensions of carotene under a variety of conditions was investigated, but the results proved, not unexpectedly, to be of no value. The next step was to attempt the direct perfusion of the intact liver with suitable carotene solutions. For this purpose healthy young adult cats were fed on a diet of cooked lean horse meat and separated milk which can be regarded as having a very low vitamin A content. By observations of the body weight and general condition of the animals it was thought that from 7 to 12 weeks was probably sufficient to deplete their reserves of vitamin, although rather wide variations were

Table II.

No. of cat	Weight of cat (kg.)	Pre-paration period (wks.)	Time of perfusion (hrs.)	Amount of carotene perfused (mg.)	Volume of perfused fluid (cc.)	Name of tissue	Weight of tissue (g.)	Weight of fat (g.)	Yellow units (10 % dil.)	Blue units (10 % dil.)	Total yellow units	Total blue units	Remarks
6	2·5	7·0	9·75	7·0	100	Liver before perfusion	4·7	0·131	6·5	Trace	128	—	No evidence of vitamin A formation
						Liver after perfusion	67·4	1·837	19·6	0·3	380	6·0	
						Blood	75·2	0·094	7·4	Trace	7	—	
						Gall bladder	1·64	0·076	Trace	0	Trace	0	
8	2·6	7·0	1·25	1·0	12	Liver before perfusion	16·4	0·318	31·4	6·2	300	64	Owing to short time of perfusion the animal may be regarded as a control
						Liver after perfusion	70·2	0·627	20·7	6·8	200	65	
						Gall bladder	2·11	0·020	Trace	0	—	0	
7	3·04	8·0	2·9	5·0	33	Liver before perfusion	6·4	2·18	7·7	29·0	270	1000	Supplies of vitamin A in the liver not exhausted; no evidence of vitamin formation
						Liver after perfusion	87·6	3·254	46·1	29·0	1600	1000	
						Blood	38·5	0·11	3·6	Trace	4	—	
						Gall bladder	1·97	0·16	Trace	Trace	—	—	
12	2·58	11·0	17·75	20·0	96	Liver before perfusion	4·3	0·108	2·6	39·4	42	640	No evidence of vitamin A formation
						Liver after perfusion	62·8	1·508	77·8	34·4	1260	560	
10	2·59	11·0	14·75	20·0	100	Liver before perfusion	3·4	0·055	7·8	380	125	6150	Doubtful evidence of vitamin A formation
						Liver after perfusion	61·7	1·550	63	545	1000	8720	

10 % colour units refer in the case of yellow units to a 10 % solution of the fat in chloroform and examined in a thickness of 1 cm.; and in the case of blue units to those obtained by mixing 0·2 cc. of a 10 % solution of the fat with 2 cc. of antimony trichloride reagent, in a 1 cm. cell, the colours being matched after 30 sec.

Total colour units are obtained by the examination of a solution of the total amount of the fat in a suitable volume of chloroform and multiplying the values so obtained with the total volume.

The red or yellow tints which were inserted to match exactly the yellow and blue colours have been omitted from the Table.

Spectroscopic examinations were made by Dr Morton for the 328 $m\mu$ band and the blue colour developed with antimony trichloride reagent.

encountered. When considered to have reached a condition of depletion the animals were placed under amytal anaesthesia, the spleen was tied off, and a small lobe of the liver was removed to provide control material[1]. A colloidal suspension of carotene in gum saline (usually 10 mg./100 cc.) was then slowly infused into the liver at the rate of approximately 10 cc. an hour, through a branch of the splenic vein. A suitable time after the infusion was completed, the animal was bled, and the remainder of the liver excised for examination. The blood and liver samples were quickly ground up with anhydrous sodium sulphate and the fats thoroughly extracted by ether. In some cases the contents of the gall bladder were also examined. The results of the examination of these fats are shown in Table II.

Cat No. 8 may be regarded as a control, for it died when it had been under experiment only for $1\frac{1}{4}$ hours during which it received 1 mg. of carotene. In none of the other experiments is there any clear evidence from the colorimetric estimations of the formation of vitamin A from carotene in the liver. Unfortunately the only experimental result tending to point in that direction (cat No. 10) is rendered questionable by the uncertainty of the estimations on the control sample of the liver tissue. On the other hand the two most satisfactory experiments (cats Nos. 6 and 12) definitely indicate that there was no conversion of carotene into vitamin A in periods of $9\frac{3}{4}$ and $17\frac{3}{4}$ hours respectively. The increase in the yellow colour of the liver fats after perfusion was not as great as might have been expected, and was at first somewhat naturally attributed to carotene. When, however, certain of the preparations were examined spectroscopically by Dr Morton it was found that the greater part of the yellow colour was due to other substances. This pointed to the pigment having undergone considerable change. But even on the assumption that all the yellow colour of the liver fat was due to carotene, there was a great discrepancy between the amounts found in the liver and that administered in the perfusion. Some of the figures are given in Table III.

Table III.

No. of cat	Time of perfusion (hrs.)	Amount of carotene given (mg.)	Carotene in the liver before perfusion (mg.)	Carotene in the liver after perfusion (mg.)	Increase in carotene (mg.)	Amount of carotene unaccounted for (mg.)
7	2·9	5	0·159	1·145	0·986	4·014
6	9·8	7	0·078	0·171	0·093	6·907
10	14·8	20	0·074	0·656	0·582	19·418
12	17·8	20	0·037	0·777	0·740	19·260

Since little or no carotene was detected in the blood and in view of the spectroscopic evidence that the yellow colour of the liver fat is largely due to substances other than carotene, it seems reasonable to conclude that most of the pigment introduced into the blood supply of the liver had been converted into other substances but not appreciably into vitamin A.

In this connection it is interesting to remember that Palmer [1922, Palmer

[1] The operations on these animals were very kindly performed by Prof. C. L. Evans.

and Kennedy, 1921] has for many years past considered the normal yellow lipochrome pigment of the mammalian liver to be a derived carotenoid. It is also appropriate to recall his repeated warnings against concluding that a pigment is carotene on the basis of solubilities and simple colour reactions. Without confirmation from a spectroscopic examination values obtained for the yellow/blue ratio should be interpreted with caution.

The failure of the perfusion experiments to demonstrate conversion of carotene into vitamin A in the livers of cats led to an attempt being made to ascertain whether the vitamin could be detected in the body a short time after the administration of the pigment by the mouth. Young adult cats were fed on the vitamin A-poor diet of cooked horse meat and separated milk. In view of the incomplete depletion of the livers in some of the former group of animals, the preliminary period for this group was fixed at 16 weeks. During this period there was fairly steady growth for the first two months, but in all cases the weight had declined considerably by the end of the four months. One cat (No. 17) was killed to serve as control, although from previous experience it was appreciated that it was a difficult matter to get adequate control material for this particular experiment. Another cat (No. 14) was deprived of food for 12 hours and then given 100 mg. of carotene dissolved in oil and then emulsified with separated milk. Six hours after the administration it was killed for examination. In a third case (Cat No. 18) two doses of 50 mg. of carotene were similarly given, one 20 hours after the other, and the animal was killed 40 hours after the first administration. The results of the examination of the materials obtained from their tissues are set out in Table IV.

The examination of the cats revealed that even after 40 hours a very small proportion of the carotene had been absorbed. By far the greater part remained in the gut in the form of an intensely pigmented fat. The pigment present in the contents of the alimentary tract was proved by spectroscopic examination to be carotene and no evidence of a formation of vitamin A was obtained.

The increase in the yellow colour of the liver-fats and possibly of the blood-fats also, of cats Nos. 14 and 18, suggested that carotene had been absorbed, and the unchanged pigment was detected spectroscopically in the liver fat of cat No. 18. There was, however, no clear evidence from either the colorimetric or spectroscopic examinations to indicate the formation of vitamin A. The liver fat of cat No. 18 showed an indication of a band at 328 $m\mu$ but it was very slight and might have represented a residual trace of vitamin from the preparatory period.

To extend this line of investigation other cats were given small doses of carotene for long periods. The dose was usually 2 mg. a day, given in the form of an oily emulsion in separated milk for periods of 8–13 weeks. Two control animals received 1 cc. of good cod-liver oil in the form of 2 cc. of a 50 % emulsion. The results are shown in Table V.

The controls grew normally and showed almost complete utilisation of the

Table IV.

(The estimations of colour etc. were made as described in the note to Table II.)

Source of fat	Experiment	Cat No. 17 (control)	Cat No. 14 (killed 6 hrs. after 100 mg. dose of carotene)	Cat No. 18 (killed 40 hrs. after two 50 mg. doses of carotene)
Liver	Weight of the tissue (g.)	74·8	96·6	54·2
	Weight of fat (g.)	5·975	5·178	1·984
	Yellow units (10 % dil.)	2·6	7·2	25·2
	Blue units (10 % dil.)	2·2	0·7	2·6
	Total yellow units	155	373	504
	Total blue units	130	37	52
	Spectroscopic examination	No evidence of carotene or 328 $m\mu$ band	No carotene or 328 $m\mu$ band	Mainly carotene, 328 $m\mu$ band doubtful
Blood	Weight of fat (g.)	0·075	0·292	0·242
	Yellow units (10 % dil.)	8·3	13·7	13·2
	Blue units (10 % dil.)	0	0	0
	Spectroscopic examination	No carotene or vitamin A detected	No carotene or vitamin A detected	No carotene or vitamin A detected
Stomach	Weight of fat (g.)	—	2·254	1·22
	Yellow units (10 % dil.)	—	1,100	170
	Blue units (10 % dil.)	—	33	7
	Total yellow units	—	25,000	2,000
	Total blue units	—	750	80
	Spectroscopic examination	—	Carotene present, but no vitamin A	Carotene present, but no vitamin A
Intestine	Weight of fat (g.)	—	0·918	1·337
	Yellow units (10 % dil.)	—	4,000	240
	Blue units (10 % dil.)	—	92	7
	Total yellow units	—	37,000	1,200
	Total blue units	—	850	90
	Spectroscopic examination	—	Carotene present, but no vitamin A	Carotene present, but no vitamin A
Caecum	Weight of fat (g.)	—	0·024	1·026
	Yellow units (10 % dil.)	—	5,200	6,900
	Blue units (10 % dil.)	—	100	100
	Total yellow units	—	1,250	71,000
	Total blue units	—	25	1,050
	Spectroscopic examination	—	Carotene, present but no vitamin A	Carotene present, but no vitamin A
Gall bladder	Weight of fat (g.)	0·006	0·018	0·017
	Yellow units	Trace	Trace	Trace
	Blue units	0	0	0

vitamin A supplied in the cod-liver oil supplement, as no trace of it could be detected in the faeces. The livers showed considerable storage of vitamin A and there were apparently traces of it present in the blood. These results are in marked contrast to those obtained on examining the cats fed on carotene, which show comparatively small vitamin deposition in the liver. In part this result was due to imperfect absorption, for during the feeding period it was

Table V.

No. of cat	Dose	Tissue	Weight of tissue (g.)	Weight of fat (g.)	Yellow units (10 % dil.)	Blue units (10 % dil.)	Total yellow units	Total blue units
4	1 cc. of cod-liver oil for 8·5 weeks	Liver	76·5	1·834	27·1	435	500	8000
		Blood	78·2	0·22	3·0	3·2	6·6	7·0
		Gall bladder	1·98	0·017	0	0	0	0
3	Ditto	Liver	75·7	1·831	26·3	210	480	3850
		Blood	94·8	0·111	5·4	Trace	6·0	—
		Gall bladder	1·62	0·023	0	0	0	0
1	2 mg. carotene for 8·5 weeks	Liver	67·7	1·734	11·6	4·0	130	70
		Blood	81·0	0·084	5·9	3·5	5·0	3·0
		Gall bladder	1·17	0·015	Trace	Trace	—	—
2	Ditto	Liver	79·7	3·452	21·4	2·5	740	90
		Blood	90·0	0·22	3·2	0	7·0	0
		Gall bladder	1·240	0·021	0	0	0	0

ascertained that the cats were excreting approximately half of the pigment administered. The absorbed portion should, however, have been more than sufficient for the requirements of the animals on the basis of comparison with rats, although the work of Capper, McKibbin and Prentice [1931] suggests that there may be considerable differences between species in respect to the amount of carotene required.

Summary.

1. The absorption of carotene from the intestinal tract may be considerably affected by the composition of the diet. Rats fed on a fat-free diet excreted as much as 90 % of the pigment when it was administered in the form of a solution in ethyl laurate. Absorption was better when the carotene was given in the form of palm oil and was nearly complete when the basal diet contained 10 % of fat. Imperfect absorption was also observed in cats fed on a diet of lean meat and separated milk.

2. Evidence was obtained of the presence of a substance resembling vitamin A in the contents of the caecum of rats fed on a diet deficient in vitamin A but supplemented with carotene.

3. Experiments were made to ascertain whether carotene can be converted into vitamin A by the action of intestinal bacteria. The great majority of these tests gave negative results but in two cases of anaerobic incubation a substance of pale yellow colour, giving a more intense reaction with antimony trichloride than the original pigment, was formed, but it was not found possible to identify it as vitamin A.

4. Attempts to convert carotene into vitamin A by incubation with liver tissue *in vitro* failed.

5. An attempt was made to ascertain whether carotene is converted in the liver into vitamin A by perfusing the organ in cats whose reserves of the vitamin had been depleted by a long period of feeding on a vitamin A-poor diet. No evidence of such conversion was obtained in periods up to $17\frac{3}{4}$ hours.

6. Administration of large doses of carotene by the mouth to cats whose reserves of vitamin A had been depleted did not result in the appearance of

vitamin A in the liver or blood up to 40 hours. A large proportion of the carotene was not absorbed but evidence was obtained to show that the pigment that entered the tissues was converted into other substances.

I wish to express my deep indebtedness to Prof. J. C. Drummond for his kind help and advice throughout the course of this investigation and to Dr R. A. Morton for carrying out the spectroscopic examination of the fats. The expenses of this investigation were defrayed from a grant of the Medical Research Council to Prof. J. C. Drummond.

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CXXXIV. THE DISTRIBUTION OF THE VITAMIN B COMPLEX.
II. ROOT VEGETABLES.

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The following work is a continuation of that already carried out in this laboratory on the estimation of the amount of the antineuritic vitamin B_1 and the antidermatitis vitamin B_2 in naturally-occurring foodstuffs [Aykroyd and Roscoe, 1929; Aykroyd, 1930; Roscoe, 1930].

Since this work was started several constituents of the vitamin B complex other than vitamins B_1 and B_2 have been differentiated by various workers. Vitamin B_3 [Williams and Waterman, 1928] has been shown to be necessary for the growth of pigeons but not for that of rats, on which these experiments have been carried out. Vitamin B_4 has been shown by Reader [1928, 1929, 1930] to be necessary for the continued growth and well-being of rats, and the possibility of its presence or absence having affected the assays of vitamins B_1 and B_2 described in this and former papers is discussed later.

Historical.

The presence of the antineuritic vitamin (B_1) in the potato was established by the early workers with pigeons. Vedder and Clark [1912] obtained partial prevention of polyneuritis with 10 g. of the fresh tuber. McCollum and Kennedy [1916] obtained a slow cure with potato juice and Chick and Hume [1917] with an alcoholic extract of the peelings. An extract of the peeled tuber improved the condition of the birds but did not cure the nervous symptoms.

A number of American workers tested various root vegetables by feeding them to rats as the sole source of the vitamin B complex [McCollum, Simmonds and Parsons, 1918; Denton and Kohman, 1918; Steenbock and Gross, 1919]. None of these tests, however, can be interpreted to show minimal amounts of the roots providing adequate supplies of either vitamin B_1 or B_2, since it must remain uncertain which of the two was acting as limiting factor. Moreover the vitamin-containing substances were fed as percentages of the diet and as will be shown later this method is not capable of quantitative interpretation.

Osborne and Mendel [1919, 1920] investigated the daily dose of roots

[1] Beit Memorial Fellow.

necessary for the normal growth of rats when supplementing a basal diet, the protein of which was an extracted meat residue. It seems probable from control experiments [Osborne and Mendel, 1917] that this residue contained vitamin B_2 but no vitamin B_1, and that their experiments may, therefore, be interpreted as vitamin B_1 estimations. They found that 1·0 g. daily dry weight of either carrot or turnip was needed for good growth, 1·0 g. dry weight of either old or new potato only provided enough vitamin for subnormal growth, 2·0 g. was enough for approximately normal growth. The peel was not found to be a better source of this vitamin than the inside. 2·0 g. of beetroot supported fairly good growth.

Dunham [1921] used the weight maintenance of 4–5 weeks old rats as criterion of "vitamin B" content. Since vitamin B_2 is not necessary for weight maintenance his results may also be interpreted as vitamin B_1 estimations. He found that 0·065 g. of dry potato, 0·07 g. of dry carrot, or 0·105 g. of turnip was needed. The lowness of his figures, as compared with those of Sherman given below, is probably due to the fact that in his experiments coprophagy was not prevented.

Sherman [1926] also quotes figures for the weight maintenance of young rats, but no experimental details are given. He reports that 3·2–3·8 g. fresh (0·32–0·38 g. dry) of carrot and 3·5 g. fresh (0·38 g. dry) of turnip and 3·5 g. fresh (0·7 g. dry) of potato were needed to maintain weight.

Morgan [1924] obtained cures of paralysis due to vitamin B_1 deficiency in rats by daily doses of 1·0 g. of dry radish. The cures and resultant growth were not quite so good as those with rats receiving 0·3 g. of dry brewer's yeast.

In the following paper the vitamin B_1 and vitamin B_2 values of potato, carrot and turnip are reported and compared with those of yeast and the other natural foodstuffs previously investigated. These vegetables were chosen as representative species of the so-called root vegetables, the potato being really an underground stem or tuber. Carrots and turnips are both roots but they differ in that the carrot is highly pigmented and the turnip is colourless. All three are among the most commonly used in ordinary diets.

Methods.

The methods employed were those described in the previous paper [Roscoe, 1930], the growth of young, 40 g., rats being used as criterion. These methods are slight modifications of those of Chick and Roscoe [1928, 1929].

The vitamin B_1 estimations were made using the unpurified caseinogen diet (FL), vitamin B_2 being supplied as autoclaved yeast extract (F5A). The vitamin B_2 estimations were made with the purified caseinogen diet (P2L) with vitamin B_1 as Peters's antineuritic concentrate from yeast (YE) [Peters, 1924; Kinnersley and Peters, 1925].

The relatively large amounts of the roots that it was necessary to administer made it impracticable to give them as daily doses. They were therefore minced and incorporated in the diet, the caseinogen and rice starch of which were varied so as to keep the percentage of protein at 20, the rest of the material being substituted for rice starch. The rats were not, however, allowed to eat more than a given amount of these diets, the remainder of their requirements being made up with FL or P2L.

Diets with the following varying percentages of incorporated vegetables were employed:

10 %	daily ration	2·5 g.	equivalent to a daily dose of		0·25 g.
10 %	„	5·0 g.	„	„	0·5 g.
20 %	„	5·0 g.	„	„	1·0 g.
40 %	„	5·0 g.	„	„	2·0 g.
65 %	„	6·0 g.	„	„	4·0 g.

These figures are for dry weights. All the diets were, however, mixed with 50 % of water. When the roots contained more water than would make up this amount they were reduced before incorporation in the diet by partial drying at 37°. All diets were cooked for 2–3 hours.

The vitamin B_1 content of root vegetables.

The results of these experiments are given in Table I. The amounts consumed daily, given in the second column, are expressed as dry weight.

Table I. *Vitamin B_1 content of root vegetables as demonstrated by weight increase of rats during five weeks on a diet, complete, except for vitamin B_1, and containing various amounts of the root vegetables to be tested.*

Material tested	Amount eaten daily, g. dry weight	No. of rats	Increase in weight in 5 weeks (averages, g.)
Carrot	0·25	6	27
	0·5	4	65
	1·0	4	88
Turnip	0·5	4	49
	1·0	5	79
Potato	1·0	4	45
	2·0	4	95

Carrot. This proved a good source of the antineuritic vitamin; rats receiving 0·5 g. gained 65 g. in weight in five weeks, those receiving 1·0 g., 88 g.

Turnip. This was a slightly less good source; rats receiving 0·5 g. gained 49 g. in five weeks, those receiving 1·0 g., 79 g.

Potato. This was a poor source of the vitamin; rats receiving 1·0 g. only increased in weight 45 g. in 5 weeks, those receiving 2·0 g. increased 95 g.

In Table III the amounts of these three root vegetables and of other foodstuffs previously tested needed to give 50–60 g. increase in weight in rats in 5 weeks when given as source of vitamin B_1 are compared and their relative potency is given, yeast being taken as 100.

It will be seen that carrot and turnip are much less potent than yeast in vitamin B_1, approximately equal to the green leafy vegetables and better than egg-yolk or meat. Potato is poorer than any of these.

Potato starch was also tested for its vitamin B_1 content, but the animals would not eat enough to provide a dose of more than 2–3 g. daily and these collapsed with paralysis at the same time as the controls. When receiving a small dose of vitamin B_1 extract, however, the animals on a diet containing potato starch grew better than those on a diet containing rice starch. There would therefore appear to be a small amount of the antineuritic vitamin in potato starch.

THE VITAMIN B_2 CONTENT OF ROOT VEGETABLES.

The results of these experiments are given in Table II. The amounts consumed daily are expressed as the dry weight.

Table II. *Vitamin B_2 content of root vegetables as demonstrated by weight increase of rats during five weeks on a diet, complete, except for vitamin B_2, and containing various percentages of the root vegetables to be tested.*

Material tested	Amount eaten daily, g. dry weight	No. of rats	Increase in weight in 5 weeks (averages, g.)
Carrot	0·5	6	48
	1·0	4	67
	2·0	4	81
Turnip	1·0	4	52
	2·0	4	62
Potato	2·0	4	38
	4·0	4	71

Carrot. This proved the richest of the root vegetables tested in vitamin B_2 as in vitamin B_1; rats receiving 0·5 g. gained 48 g. in 5 weeks, those receiving 1·0 g., 67 g., and those with 2·0 g., 81 g.

Turnip. This was less rich; rats receiving 1·0 g. gained 52 g. and those receiving 2·0 g., 62 g.

Potato. This was a poor source of the vitamin; rats receiving as much as 2·0 g. gained only 38 g. but those with 4·0 g. gained 71 g. during the five weeks of the experiment.

In Table III these vegetables and other foodstuffs are compared with yeast

Table III. *The vitamin B_1 and B_2 content of various foodstuffs compared with that of yeast.*

In columns 1, 2, 4 and 5 are given the minimal amounts needed to promote an increase in weight of 50–60 g. in 5 weeks in young rats of 40 g. weight when given as source of vitamins B_1 and B_2 respectively.

In columns 3 and 6 are shown the relative potencies of the dried foodstuffs when yeast is taken as 100.

		As source of vitamin B_1			As source of vitamin B_2		
	Water content	Weight increase		Potency	Weight increase		Potency
		Wet wt.	Dry wt.	Dry wt.	Wet wt.	Dry wt.	Dry wt.
Material	(%)	1	2	3	4	5	6
Yeast	80	0·25	0·05	100	0·5	0·1	100
Green vegetables	90	3·0–5·0	0·3–0·5	10–17	4·0–6·0	0·4–0·6	17–25
Carrot	90	2·5–5·0	0·25–0·5	10–20	>5·0	>0·5	<20
Turnip	93	>7·0	>0·5	<10	14·3	1·0	10
Egg-yolk	50	<2·0	<1·0	>5	2·0	1·0	10
Meat, steak	75	4·0	1·0	5	2·8	0·7	14
Milk	88	—	—	—	6·0	0·7	14
Potato	78	5·0–10·0	1·0–2·0	<5	10·0–20·0	2·0–4·0	<5

as regards their vitamin B_2 potency, on the basis of the amounts needed of each to promote 50–60 g. increase in weight in 5 weeks.

All are of much lower potency than yeast; carrot has a potency equal to

that of milk, meat and the less good green vegetables, but better than egg-yolk or wheat germ. Turnip is equal to the latter group. Potato is of the same order of strength as the cereals and pulses.

Potato starch was also tested for its vitamin B_2 content. Two rats receiving 60 % potato starch diet and eating such an amount that they received an average of 2·2 and 2·6 g. of starch daily grew better than the controls in the same litter though not better than controls sometimes grow. A very low content of this vitamin is indicated.

Experiments were carried out in which these vegetables were used in the cure of dermatitis arising from a deficiency of vitamin B_2. 0·5 g. of dry carrot, turnip or potato was found to cure symptoms. The minimal amounts needed were not discovered.

The above experiments were carried on for 5 weeks only, but additional experiments were carried out in which the growth to normal adult weight, when the vegetable was the sole source of the vitamin B complex, was investigated.

Three rats, a male and two females, when receiving 2·0–3·0 g. daily of dry carrot followed the normal growth curve. A litter was born to each of the females but only one of the young survived: this one was reared to weaning.

Similarly with 4·0–8·0 g. of potato normal growth curves were obtained and each rat produced a litter. One litter of six was reared to weaning, the other, however, was not fed.

Continued breeding was not investigated.

The effect of the age of the root on the vitamin B content.

A comparison of the vitamin B_1 and vitamin B_2 contents of old carrots which had been stored all winter with those of fresh spring carrots was made. No significant difference was observed. In the vitamin B_1 tests, rats which received 0·25 g. of old carrot increased in weight an average of 29 g. in the 5 weeks of the experiment, those receiving the new carrot 20 g. In the vitamin B_2 tests those receiving 0·5 g. of old carrot increased 42 g. in weight, those with the same addition of new carrot 44 g.

This result recalls that of Osborne and Mendel [1920] who found new potatoes not to be superior in "water-soluble B" to old ones.

The effect of cooking on the availability of the B-vitamins.

In the previous experiments the cooking of the diets in which the vitamin-containing substances were included involved the steaming of the vegetables for 2–3 hours. It was thought possible that this cooking might render them more digestible and so increase the degree of absorption of the B-vitamins from the alimentary canal of the rat. An experiment was therefore performed in which the same quantities of carrot were fed (i) as a percentage of the diet which was cooked for 2–3 hours and (ii) as daily doses of the uncooked vegetable. Vitamin B_1 and vitamin B_2 tests were carried out in this way.

No difference was observable between the two groups. In the vitamin B_1 tests the average increase in weight in 5 weeks with 0·25 g. of cooked carrot was 31 g.; with the same amount of uncooked carrot it was 29 g. In the vitamin B_2 tests with 0·5 g. of cooked carrot there was an increase in weight of 47 g., with the uncooked of 42 g.

The vitamin B_4 content of the vegetables.

Vitamin B_4 deficiency in rats is characterised by failure to grow after about 4 weeks of deprivation and in acute stages by listlessness, a hunched attitude, and inflamed and swollen paws [Reader, 1928, 1929, 1930]. These symptoms have been observed in these experiments among the negative control rats which were receiving vitamin B_2 as autoclaved yeast extract together with a diet containing unpurified caseinogen. It is therefore clear that vitamin B_4 is not present in the basal diet or in the source of vitamin B_2 given in these vitamin B_1 tests. Thus it is possible that these and previous vitamin B_1 estimations may have been affected by a deficiency of vitamin B_4.

Vitamins B_1 and B_4 are, however, very closely related and there is as yet no evidence that they have a different distribution in nature. Moreover in the experiments here reported and in the majority of the previous experiments, normal growth (14–18 g. per week) was obtained with the higher doses at a time when the controls were showing symptoms of vitamin B_4 deficiency; in these experiments therefore vitamin B_4 must have been present in adequate amounts.

A further test was made in which rats on small doses of carrot, after several weeks of subnormal growth, received in addition the preparation of vitamin B_4 described by Reader [1930]. No improvement in growth was observed, showing that the limiting factor was not a deficiency of vitamin B_4.

It may therefore be taken as proved that vitamin B_4 was present in amounts sufficient for normal growth in the vegetables here tested.

In the vitamin B_2 tests vitamin B_4 is present in adequate amounts in the antineuritic yeast concentrate made according to the method of Peters, the charcoal being extracted with acid alcohol.

Discussion.

The vitamin B_1 and vitamin B_2 contents of the root vegetables appear from these results to vary considerably, carrot being a good source of both the vitamins, potato a poor one and turnip slightly poorer than carrot.

The proportion of vitamin B_1 to vitamin B_2 is similar to that found in the majority of the leafy vegetables, twice as much of the material being needed for good growth when given as source of vitamin B_2 as when given as source of vitamin B_1. This is the same proportion as is also found in brewer's yeast.

These results confirm the conclusions of all investigators in showing that carrot is a fairly good source of the B-vitamins and potato a poor one, and more

particularly they agree with those of Osborne and Mendel [1919, 1920] in showing that 1·0 g. of dry carrot and 2·0 g. of potato contain sufficient vitamin B_1 for normal growth of rats. In disagreement with the present work these authors claimed that turnip was as good a source of the antineuritic vitamin as carrot. Beyond this it is difficult to compare the results here obtained with those of previous workers.

The practice of administering the vitamin-containing substance as a percentage of the diet which is freely consumed by the experimental animal, makes the interpretation of many early results very difficult. In vitamin B_1 tests particularly this method is very uncertain in its results.

In such experiments it is possible by careful measurement of the food consumed to assess the daily intake of the vitamin-containing substance, but it is not possible, except by limiting the total intake, to insure that each animal on a given diet shall eat the same amount. Aykroyd has shown (unpublished results) that on a test diet containing a percentage of a substance low in vitamin B_1 some rats may consume only a small amount of food, and as a result do not obtain enough vitamin B_1 to protect them against paralysis, while others eat about twice as much of the diet and thereby acquire enough of the vitamin to increase their weight by 10–13 g. a week. In fact in one case a rat at first grew 10 g. a week and then ceasing to eat well lost weight and developed paralysis.

This fact would seem to disprove, as far as vitamin B_1 is concerned, the theory put forward by Plimmer, Rosedale and Raymond [1927] that the amount of vitamins B_1 and B_2 (no distinction between the two vitamins is made by these authors) needed is directly proportional to the calories consumed. For in experiments such as the above where the vitamin-containing substance is given as a percentage of the diet the proportion of vitamin to calories ingested is constant whatever the amount eaten, yet the deficiency of a rat which develops paralysis must be considered greater than that of one which shows an increase in weight of 10–13 g. a week.

A further fact indicating that the need for either vitamin B_1 or vitamin B_2 is not dependent on the calorie intake is that at the end of the five weeks tests for either of these vitamins the rats were in some cases eating twice as much food as at the beginning, yet although their vitamin supply had not been increased their growth was just as good.

Summary.

1. The vitamin B_1 content of carrot is about one-fifth that of yeast (dry weights), equal to that of the less good leafy vegetables and better than that of egg-yolk or milk. Turnip is not quite so rich in the vitamin, and potato is poor, being only one-fifteenth as rich as yeast.

2. Carrot is one-fifth as good a source of vitamin B_2 as yeast and about equal to milk, meat and the less good leafy vegetables. Turnip is less potent,

having the same potency as egg-yolk and wheat germ. Potato has a low content of this vitamin, comparable with that of the cereals and pulses, but the relatively large amounts of this vegetable eaten may render it an important source of both the B-vitamins in an ordinary diet.

3. Potato starch contains small amounts of both vitamins, B_1 and B_2.

4. New spring carrots contain no more vitamin B_1 or B_2 than do old ones which have been stored all winter.

5. The vitamins B_1 and B_2 in carrots are not rendered more available to the animal consuming them by steaming for two to three hours.

6. Reasons are given indicating the presence of vitamin B_4 in the vegetables tested.

7. The method of testing for the B-vitamins by incorporation of the test material in the basal diet is criticised and it is pointed out that this method is not admissible unless the consumption of the diet is limited.

8. The vitamin B_1 requirements of the rat do not seem to be related to the total amount of food consumed.

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CXXXV. A DIFFERENTIAL RESISTANCE THERMOMETER FOR BIOLOGICAL USE[1].

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The need has arisen for a micro-calorimeter suitable for the measurement of heat-production in biological material dissolved or suspended in a small amount of liquid. With this object in view a differential resistance thermometer of the type used by Watson and Henderson [1928] to measure the heating effects of thorium products has been constructed. The following is a description of an apparatus designed in order to measure the heat produced by about 1 g. of resting anaerobic muscle in about 2 cc. of Ringer's solution. The details of the arrangements could readily be altered and made suitable for many other purposes.

Principle of the method.

The resistance of a metal to an electric current varies as the temperature of the metal is altered. A, B, C and D in Fig. 1 are the resistances of the arms of

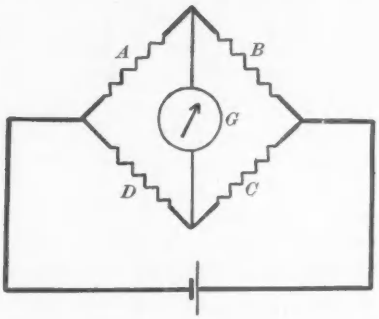

Fig. 1.

a Wheatstone bridge. When the relationship $AC = BD$ holds good, no current passes through the galvanometer G. If now the opposite arms B and D be heated, their resistance will alter and the bridge will no longer be balanced. This change in balance is the basis of the method. It is not measured directly

[1] A brief account of this was given before the Biochemical Society, but a more detailed description seems desirable.

[2] Beit Memorial Research Fellows.

by galvanometer deflection, but the bridge is re-balanced by means of an external variable resistance in parallel with one of the other two arms. The value of this compensating resistance which is necessary to restore the equilibrium of the bridge gives a measure of the heat which has been produced.

Details of arrangement.

In the actual apparatus (Fig. 2) each of the four arms of the Wheatstone bridge consists of No. 47 s.w.g. double silk-covered copper wire, having a resistance of about 200 ohms.

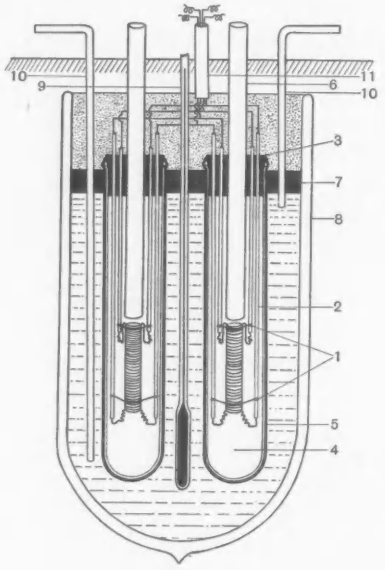

Fig. 2.

Arms *A* and *C* are wound on a silver-plated copper tube 7 cm. long and 1 cm. in diameter, with walls 0·15 mm. thick. By means of rubber bands and silk threads (1) this is mounted upon glass tubes (2) which carry the leads from the coils through a rubber stopper (3). This is then placed in a paraffined glass test-tube (4) which is enveloped in a portion of the inner tube of a bicycle tyre (5). Through the rubber stopper a glass tube (6) passes, whose diameter is slightly greater than that of the metal tube.

Arms *B* and *D* of the bridge are arranged in exactly the same manner in another similar paraffined tube. Both of the large test-tubes are sealed into a cork (7) which fits into a silvered Dewar flask (8) 5″ in diameter and 10″ deep. Passing through this cork into the interior of the Dewar flask are a high-sensitivity mercury thermometer (9) and two glass tubes (10).

Great care is taken to ensure that all the joints between test-tubes and cork and cork and Dewar flask are completely watertight, and this is especially important with regard to the fitting of the rubber stoppers into the test-tubes. The presence of the merest traces of water inside the tubes is sufficient to render the apparatus useless.

The space between the corks and the rim of the Dewar flask is filled with paraffin wax of melting-point 50°. In this paraffin are embedded the soldered connections completing the Wheatstone bridge. The four leads from the bridge are taken off through a glass tube (11) which is also embedded in the paraffin. The Dewar flask with its contents is then placed in paraffin wax in a cylindrical zinc container, and the whole is immersed in a constant temperature water-bath so that the only portions protruding from the water are the glass tubes (6) by means of which material can be lowered into the metal tubes; the glass tubes (10) by which water of the desired temperature can be introduced into the flask; and the glass tube (11) through which the copper leads pass.

The arrangement of the electrical connections to the four resistances A, B, C and D is shown in Fig. 3. As already indicated, the resistances are soldered together in the form of a Wheatstone bridge. Two leads go to a 2 v. accumulator (Ac); the circuit can be opened or closed by the switch (S_1). One accumulator lead passes through a milliammeter (MA), and the other through a variable resistance (R_1) by means of which the current through the bridge can be regulated accurately. The two galvanometer leads pass through a reversing switch (S_2). One of them also goes through a copper tap key (T) which when closed connects the galvanometer to the bridge circuit, and when open completes the circuit of a galvanometer damping resistance (R_2). Another variable resistance (R_3) is a shunt resistance in parallel with resistance C. The resistances A, B, C and D are made of such magnitude that when a current is passing through the bridge and the shunt resistance is set at about 9000 ohms, no current goes through the galvanometer. This variable resistance (R_3) is capable of accurate adjustment to 0·1 ohm.

The instrument is calibrated by the production of a known amount of heat, for which purpose a small heating coil is constructed and mounted as shown in Fig. 4. The coil (H) is made of cotton-covered constantan wire of 46 S.W.G., and has a total resistance of 32·10 ohms. It is sealed into a small glass bulb (Z), through the capillary neck of which pass leads of No. 47 S.W.G. copper which are soldered to the heating coil. On emerging from the capillary, these leads are in turn soldered to 24 S.W.G. copper wires passing through a rubber bung, by which the weight of the whole contrivance is supported. The capillary and bulb are attached to another rubber stopper which is of such a size as to fit exactly into a small glass tube (V), which in turn slips into the metal tube around which the resistances B and D are wound. The tube (V) and its contents can be lowered through the glass tube (6) in Fig. 2.

A duplicate heating coil *etc.* is made for the other metal tube. The function

of this duplicate coil is merely that of keeping the heat capacity of both halves of the Wheatstone bridge the same, and for this reason it is not necessary that it be constructed with such care. The copper leads from the duplicate are not attached to anything.

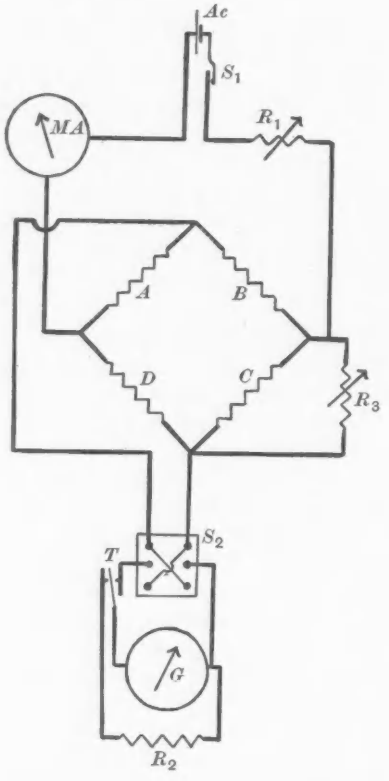

Fig. 3.

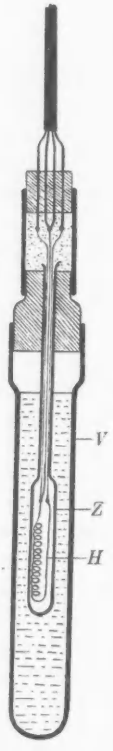

Fig. 4.

The connections to the heating coil are made as shown in Fig. 5. H is the heating coil, and L_1 its leads of 47 s.w.g. copper. R is a constantan resistance equal to the coil resistance, and L_2 are dummy leads of No. 47 s.w.g. copper, whose resistance is the same as that of L_1. These dummy leads in the actual apparatus are enclosed in the bulb and capillary together with the heating coil and its leads. P is a potentiometer, S a copper switch, W a slide-wire, and Ac a 2 v. accumulator. The position of the sliding contact T on the wire W governs the amount of current passing through H.

When points 1 and 2 on the switch are connected to points 3 and 4 respectively a current passes through H, and the amount of this current is known

by measuring the potential difference between the ends of the resistance. The measurement is made on the potentiometer, which in this position of the switch is connected to the heating coil. If points 1 and 2 are connected to points 5 and 6, the potentiometer is no longer connected to the heating coil. The

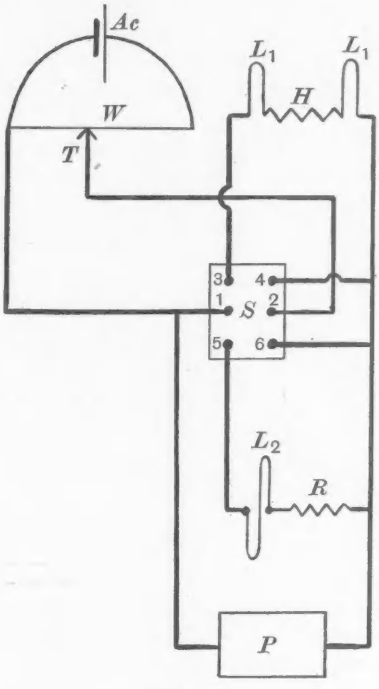

Fig. 5.

current from the slide-wire passes, under these circumstances, through an external resistance R equal to H, and then through the dummy leads L_2; it does not, however, actually pass through the heating element H.

The galvanometer in use is a moving-coil instrument made by the Cambridge Instrument Company. It has an internal resistance of 14·6 ohms, and at 1 m. scale distance gives a deflection of 600 mm. per milliamp. It can be read to 0·2 mm. at a scale distance of 2 m.

Constant temperature water-bath. The bath is essentially the same as that used by Meyerhof. It has been described by Blaschko [1928]. Small alterations have been described by Meyerhof, McCullagh and Schulz [1930]. The temperature of the bath is constant to within 0·002°.

Procedure.

Calibration. The current across the bridge is adjusted to 4 milliamp. and the temperature of the water in the Dewar flask brought to equilibrium with that in the bath by passing water through the glass tubes (10, Fig. 2).

When a large series of experiments is being carried out over a period of several days, the bridge current and the automatic thermoregulator are permitted to run continuously. This results in a constant zero, *i.e.* the amount of resistance in the shunt required to balance the bridge is in this way kept at the same value.

Although the whole system is made of copper, certain unavoidable thermo-electric effects arise. These are eliminated by averaging the readings obtained when the galvanometer current is direct and when it is reversed.

3 cc. of water are placed in each of the two small glass tubes V (see Fig. 4 and text). The heating coil and the duplicate are placed firmly into the tubes, which by means of the leads are then lowered through the glass tubes ((6), Fig. 2) into their positions in proximity to the resistances A and C, and B and D, of the Wheatstone bridge. When the system has attained thermal equilibrium, as indicated by the amount of shunt resistance required to balance the bridge remaining constant, the value of this resistance is noted. This gives the zero point. It is affected very slightly by changes in room temperature; the error due to this is constant and is allowed for. A current is now passed through the heating coil (H, Figs. 4 and 5) and the potential difference between the ends of H is determined.

From the two equations

$$i = E/R \quad \text{and} \quad C = 0{\cdot}239 i^2 R t,$$

where i = current in ampères, E = potential difference in volts, R = resistance in ohms, t = time in seconds, and C = heat in gram calories during time t, we obtain

$$C = \frac{0{\cdot}239\, E^2 t}{R}.$$

In the case of the apparatus being described the resistance of the heating coil is 32·10 ohms, and we wish to express the heat as gram calories per hour, hence

$$\text{Gram calories per hour} = \frac{0{\cdot}239 \times 3600 \times E^2}{32.10}$$
$$= 26{\cdot}8\, E^2.$$

Thus, if a current is passing through the heating coil and the potentiometer reading is 0·100, the heat production is $26{\cdot}8 \times (0{\cdot}1)^2$, or 0·268 g. cal./hour.

After the heating current has been passed for about 30 minutes the system comes to an equilibrium, as the heat loss is equal to the heat production. Two arms of the bridge are now at a higher temperature than the other two arms and for this reason the shunt resistance will have to be adjusted to a new value in order to bring the galvanometer to zero. In this manner the amount of resistance required in the shunt to balance the bridge can be ascertained for any heat production.

The heat produced by a known weight of muscle can now be measured very rapidly. The glass tube containing the heating coil is withdrawn, and the water is removed and replaced by the muscle, the weight of which should be of the order of 1 g. Ringer's solution is added in such quantity as will bring the heat capacity to its original value. (The specific heat of the muscle is about 0·825, and that of Ringer's solution about 0·995.) The heating coil is replaced, and the whole, after being cooled to a temperature rather below that of the bath, is again lowered into the apparatus. The attainment of the equilibrium condition is hastened by the use of the heating coil until the bridge is obviously approaching its state of balance. When the balance is very nearly reached the switch (*S*, Fig. 5) is put in the position where points (1) and (2) are in contact with points (5) and (6). The sliding contact *T* is then adjusted to the position it would occupy if the amount of heat being produced by the muscle were being produced by the heating coil. This results in the passage of a current through the dummy leads of the coil, of the same magnitude as that which passes through the actual leads during calibration. Thus the heat production in the leads need receive no other consideration.

The heat production of the muscle is known from the amount of resistance necessary in the shunt to balance the bridge after complete equilibrium has been established.

In some cases it is simpler to calibrate the apparatus after the experiment. If the muscle is not required for subsequent chemical measurements it can be killed by placing the small tube containing it in boiling water. The tube and contents are then cooled, dried with ether and replaced in the apparatus. After complete equilibrium has again been reached the changes in resistance which occurred during the experiment are reproduced by passing a suitable current through the heating coil.

Sensitivity. A heat production in 3 cc. of water of 1 cal. per hour results in the shunt resistance having to be changed 145·2 ohms in order to balance the bridge. The bridge can be balanced accurately to 0·1 ohm. It can be shown mathematically that 0·1 ohm. represents a temperature change of about $3° \times 10^{-5}$. By increasing the bridge current or amplifying the galvanometer current the sensitivity could be greatly increased.

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CXXXVI. STUDIES ON "NON-IRRITABLE" MUSCLE.

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The condition known as "non-irritability" or "inexcitability," as distinct from fatigue, in amphibian muscle is one to which attention was drawn by Fletcher and Hopkins [1907], though Hermann [1867] had made observations which indicated that the phenomenon had to some extent fallen under his notice. In the course of the classical experiments of Fletcher and Hopkins these authors observed that occasionally the frog muscle that they were using ceased to be irritable, in spite of the absence of rigor and the fact that the lactic acid content was not at its maximum, and that the condition was therefore not one of ordinary fatigue. The nature of the phenomenon was not further studied at the time, and it was not until 14 years later that the work of Foster and Moyle [1921] was published. In their paper it was shown that the condition of non-irritability could readily be produced in frog's muscle by keeping it at a temperature of 0° in an atmosphere of oxygen, air or nitrogen, the time necessary varying according to the gaseous medium employed. Two or three days were sufficient in the case of nitrogen, whereas two or three weeks were required for oxygen, whole limbs being used in both cases. When oxygen was used there was no rigidity at the end of the period, a circumstance which is in conformity with the experiences of Fletcher and Hopkins.

Foster and Moyle then made an investigation of some of the chemical properties of amphibian muscle in the non-irritable state, and they found that heat, toluene, and mechanical injury all have the same effect as in the case of normal muscle; that is to say, all these treatments result in the production of large amounts of lactic acid. An extension of these studies to some of the physical properties led to the conclusion that the loss of irritability is associated with a redistribution of certain ions; perhaps as a result of changes in membrane permeability, or perhaps caused by adsorption on colloidal surfaces. This "ionic" theory was supported by the very important further observation that the loss of irritability was reversible; that by treating the inexcitable muscle with various solutions it was possible to recover a certain degree of irritability. Thomson's [1928] experiments on the internal conductivity of normal and non-irritable muscle, demonstrating as they do a drop

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of over 40 % in respect of the latter, furnish additional evidence that a disappearance of free ions is involved in the causation of the phenomenon. Dulière and Horton [1928], pursuing an investigation of non-irritable frog's sartorii, came to the conclusion that the loss of excitability was dependent upon the diffusion of potassium ions from the interior of the fibres to the interspaces between them.

There thus appears to be a fair accumulation of data which support the hypothesis of what Foster and Moyle called the "ionic" origin of non-irritability in muscle. Whether or not this be accepted, however, a further study of the phenomenon appealed to the present authors from the following standpoint. In view of the fact that the muscle can be restored to its original condition by immersion in certain solutions, it is obvious that it is not in any way irreversibly damaged; we have, in the circumstance that we can at will suppress the excitability of a muscle and at will restore it again, a means ready to hand of eliminating certain phases of the activity of the tissue, thereby leaving the way more open for a study of those that remain. The series of metabolic and energy-producing changes in muscle is so complicated that any technique by means of which it is possible to attain even a partial separation of these processes must be a valuable one. Any addition to the array of facts already discovered in connection with non-irritable muscle may therefore be of importance with regard to elucidation of the processes occurring in normal muscle.

The work to be described in the present paper may conveniently be divided into four sections, *viz.* A. The chemical effects of stimulation of non-irritable muscle; B. The thermal effects of stimulation of non-irritable muscle; C. The resting heat-rate of non-irritable muscle; and D. The caloric quotient of non-irritable muscle.

A. The chemical effects of stimulation of non-irritable muscle.

As has been mentioned above, Foster and Moyle [1921] came to the conclusion that there are "no chemical differences" between non-irritable and normal muscle. They stated that "non-irritable muscle formed lactic acid in exactly the same circumstances as fresh irritable muscle," by which they meant that the effect of heating the muscle, treating it with toluene, or subjecting it to mechanical injury was to give rise to the production of lactic acid whether the muscle was normal or non-irritable. Curiously enough, neither these nor any other workers tried the experiment of directly or indirectly stimulating the muscle electrically, in order to see whether any lactic acid was then produced. To do this becomes of importance, when it is considered that the mechanism of irritability is far from being understood. The fact that a non-irritable muscle gives no contraction on stimulation may be due to one of at least two causes, namely that (*a*) the mechanism by which lactic acid is formed from carbohydrate as a consequence of stimulation may be inhibited, or (*b*) lactic acid may still be produced in the ordinary way, but

may be unable to bring about any response in the muscle on account of derangement of the contractile mechanism.

Methods.

(i) *Production of non-irritability in muscles.* A number of skinned hind limb-pairs from frogs were suspended at 0° in a large closed jar, at the bottom of which was a layer of water, out of contact with the limbs, but whose presence was necessary to prevent desiccation of the muscles. Two limb-pairs were connected to silver wires leading outside the jar and were used as tests for irritability, but were not employed in the actual experiments. It was usually found that from 4 days to a week in air produced non-irritability, and that no bacterial or other contamination made its appearance in that time; nor was there any trace of rigidity.

(ii) *Recovery of irritability.* Non-irritable limbs were completely immersed in amphibian Ringer's solution, through which a current of oxygen was continuously bubbled. Excitability began to return as a rule within 20 minutes, as evinced by the response of the muscles to single induction shocks, and after an hour or two's treatment the muscles were in most cases strongly irritable. When the muscles were to be used for subsequent experimentation, it was naturally undesirable to give more shocks than could be helped, for fear of vitiating the results by causing accumulation of lactic acid.

(iii) *Estimation of lactic acid.* The muscles were dissected off the limbs as rapidly as possible on a cold plate and immediately ground in an ice-cooled mortar with sand and 5 cc. of ice-cold 20 % trichloroacetic acid. The pulp was washed into a measuring flask with further trichloroacetic acid and was made up with water to a total volume of 50 cc. Having stood overnight in the ice-chest, the contents of the flask were filtered, neutralised with saturated potassium hydroxide, and interfering carbohydrates removed by the usual copper-lime method. After filtering again, lactic acid was estimated by the method of Friedemann, Cotonio and Shaffer [1927].

(iv) *Estimation of total carbohydrates.* This was performed by the method described by Lohmann [1928].

Results.

Two non-irritable limb-pairs were weighed. All the muscles were then dissected off both right legs and immediately worked up as described above for lactic acid estimation. The limb-pairs were meanwhile weighed again, and the muscles of the remaining legs were stimulated for 10 minutes with continuous induction shocks. These muscles were at once dissected away after the cessation of stimulation and were also treated with trichloroacetic acid. The limb-skeletons were re-weighed; by these successive weighings the amount of muscle taken before and after stimulation was known. Two limb-pairs which had been rendered non-irritable in the same batch as the above two were allowed to regain their irritability in oxygenated Ringer's solution; they then underwent the same sequence of weighing, dissection and stimulation.

The results of the lactic acid estimations are comprised in the first experiment recorded in Table I, which contains also the protocols of other experiments of the same kind made at different times.

Table I.

			Weight of muscle (g.)	Mg. lactic acid per g.	Change in lactic acid content on stimulation
1.	Non-irritable	Right legs	4·350	0·77	—
		Left „	4·581	0·76	−0·01
	Recovered	Right „	3·118	0·28	—
		Left „	2·930	0·49	+0·21
2.	Non-irritable	Right legs	4·59	0·90	—
		Left „	4·72	1·00	+0·10
	Recovered	Right „	5·85	0·65	—
		Left „	5·80	0·89	+0·24
*3.	Non-irritable	Right legs	5·732	0·87	—
		Left „	5·540	0·95	+0·08
	Recovered	Right „	6·555	0·54	—
		Left „	6·391	0·90	+0·36
*4.	Non-irritable	Right legs	4·224	0·65	—
		Left „	4·451	0·62	−0·03
	Recovered	Right „	5·120	0·38	—
		Left „	5·340	0·64	+0·26
5.	Non-irritable	Right legs	4·805	0·88	—
		Left „	4·721	0·93	+0·05
	Recovered	Right „	5·004	0·58	—
		Left „	5·182	0·99	+0·41

* In these experiments the stimulation was direct; in the others it was indirect, *i.e. via* the nerves.

It is noticeable that the lactic acid content of the recovered muscles is always lower than that of the non-irritable ones. This can be ascribed partly to the diffusion of lactic acid in the Ringer's solution, and partly to a certain amount of oxidative resynthesis during recovery.

From these results the conclusion can be drawn that, although in non-irritable muscle the lactic acid-forming mechanism exists and, as Foster and Moyle showed, is able to function under a variety of conditions, nevertheless the ordinary type of stimulation is unable to bring it into play. What has been called the "trigger mechanism" seems to be incapacitated in some way, and the stimulus is unable to initiate the series of reactions leading to the production of lactic acid.

There remains however the possibility that although lactic acid does not appear, carbohydrates are nevertheless disappearing under the influence of the stimulation, but are undergoing some other transformation. To test this point it was necessary to repeat the above experiments, with the difference that instead of lactic acid, total carbohydrates were estimated before and after stimulation.

The results obtained are given in Table II.

Table II.

			Weight of muscle (g.)	Mg. carbohydrate per g.	Change in carbohydrate on stimulation
1.	Non-irritable	Right legs	4·72	1·58	—
		Left ,,	4·66	1·62	+0·04
	Recovered	Right ,,	4·90	2·01	—
		Left ,,	5·25	1·88	−0·13
2.	Non-irritable	Right legs	3·68	1·70	—
		Left ,,	3·51	1·67	−0·03
	Recovered	Right ,,	4·04	2·54	—
		Left ,,	4·19	2·24	−0·30
*3.	Non-irritable	Right legs	4·83	1·76	—
		Left ,,	4·88	1·74	−0·02
	Recovered	Right ,,	3·75	2·63	—
		Left ,,	3·92	2·35	−0·28
4.	Non-irritable	Right legs	3·50	1·43	—
		Left ,,	3·61	1·45	+0·02
	Recovered	Right ,,	4·21	2·10	—
		Left ,,	3·99	1·84	−0·26

* In this experiment direct stimulation was employed.

It is evident from this that in addition to there being no lactic acid production upon stimulation of non-irritable muscle, there is no disappearance of carbohydrate. After recovery in oxygenated Ringer's solution, the muscle is restored to its normal state and stimulation leads to the production of lactic acid and the disappearance of carbohydrate in the usual manner. The conclusion appears justifiable then that the phenomenon of non-irritability has its rise in a failure to act of the so-called "trigger mechanism" and not in a failure of the contractile mechanism to respond. The observation of Foster and Moyle, that stimulation of a non-irritable muscle produces no action current, is worthy of notice in this regard, for there is reason to suppose that the action current is a phenomenon associated with excitation and not with contraction.

B. The thermal effects of stimulation of non-irritable muscle.

For the purposes of studying this question, use was made of the differential resistance thermometer described by McCullagh and Case [1931]. The gastrocnemius of the frog was the muscle employed, and it was dissected out from the non-irritable limb together with the sciatic nerve. The muscle was placed in the tube V [1931, Fig. 4] containing the heating coil, together with distilled water in such amount that the total heat capacity was equal to that of 3 cc. of water. (Ringer's solution could obviously not be used, as it would tend to bring about restoration of the muscle.) The nerve passed through a slot in the stopper of the tube V and lay across two small closely-placed electrodes of silver wire, which in turn were connected to two copper wires which passed up the tube 6 and thence to an induction coil. By this means the muscle could, when desired,

be stimulated *in situ* in the calorimeter without disturbing any part of the apparatus.

The technique of using the apparatus has been fully described in the previous paper and need not be enlarged upon here. After the bridge had attained equilibrium the muscle was stimulated for ten successive periods of 10 seconds, with 10-second intervals, using rapidly-recurring faradic currents from a coil. The balancing resistance values were noted at regular intervals for about an hour, at the end of which period the reading had returned to its original value. The tube containing the muscle was now removed and placed in a beaker of boiling water for 10 minutes, cooled, dried, and put back into the apparatus. When equilibrium was again reached, the stimulation from the coil was repeated in exactly the same manner as before, and fresh readings were taken. The reason for this procedure was that the passage of the stimulating current between the electrodes, although these were not actually enclosed in the tube V, might be expected to give rise to some heat production of a purely physical nature, which would be of the same magnitude whether the muscle were alive or had been killed by exposure to the temperature of boiling water.

Table III.

	Readings of shunt resistance (ohms)	
	Non-irritable muscle	Same muscle after heating
1. Equilibrium	8327·8	8327·9
5 mins. after stimulation	8328·3	8328·2
10 " "	8328·9	8328·9
15 " "	8329·2	8329·1
20 " "	8329·2	8329·2
25 " "	8329·1	8329·2
30 " "	8329·0	8329·1
35 " "	8328·8	8328·8
40 " "	8328·3	8328·2
45 " "	8328·1	8328·2
50 " "	8327·9	8328·0
55 " "	8327·8	8327·7
60 " "	8327·9	8327·8
	Constant	Constant
2. Equilibrium	8553·6	8553·4
5 mins. after stimulation	8554·0	8553·9
10 " "	8554·7	8554·6
15 " "	8554·9	8550·0
20 " "	8554·9	8554·8
25 " "	8554·8	8554·8
30 " "	8554·6	8554·7
35 " "	8554·4	8554·5
40 " "	8554·1	8554·2
45 " "	8553·9	8554·0
50 " "	8553·7	8553·7
55 " "	8553·7	8553·6
60 " "	8553·7	8553·5
	Constant	Constant

In all the experiments that were made, it was found that the heat production upon stimulation was extremely small, and moreover that it was not

altered after the muscle had been heated at 100° for 10 minutes. The slight heat changes that did occur, therefore, were to be ascribed to the physical effects of the passage of the current through the electrodes and nerve, and not to any chemical processes taking place in the muscle as a result of stimulation. This finding, that stimulation of non-irritable muscle leads to no heat production, was not unexpected in view of the chemical experiments already reported.

Some typical figures for these thermal experiments are given in Table III.

It is seen that the changes in resistance, which in themselves are very small, run so closely parallel in the cases of the unheated and the heated non-irritable muscle that there can be no doubt that they are not to be attributed to any physiological heat production in the muscle.

C. The resting heat-rate of non-irritable muscle.

A determination of the resting heat-rate of non-irritable muscle under aerobic and anaerobic conditions would be expected to give some indication as to whether the metabolic processes are quantitatively different from those in normal muscle, and as to whether the oxidative reactions are still going on or are suppressed. Several experiments were performed in order to obtain values for the resting heat production, using the differential resistance thermometer and employing the technique described in the previous paper.

The muscle was dissected as carefully as possible from a non-irritable limb, which was weighed before and after dissection. The known weight of muscle was placed in the tube V of the apparatus together with enough distilled water to bring the heat capacity to that of 3 cc. of water. The necessary balancing resistance was determined in the usual way, after which the air in the tube was replaced by nitrogen, this gas being bubbled through the water also for several minutes before re-closing the tube and inserting it in the apparatus. The shunt resistance at equilibrium was again determined. Finally, the tube containing the muscle was placed in a beaker of boiling water for 10 minutes in order to kill the muscle, was dried, cooled, and replaced. A current was passed through the heating coil of such magnitude as to necessitate the same alteration in the shunt resistance as was found necessary during the anaerobic experiment with the live muscle, after which the current was increased so as to obtain the value for the aerobic half of the experiment. The potentiometer readings were taken in both cases, and the heat production calculated from the formula given in the preceding paper.

The figures for the different experiments are set forth in Table IV.

It follows from these figures that the resting anaerobic heat-rate of non-irritable muscle is in the neighbourhood of 0·094 g. cals. per g. of tissue per hour. (This is the mean value of the seven recorded.) The observed variation between 0·080 and 0·117 is by no means larger than is usually encountered in determinations of this nature. The figure 0·094 is in remarkably good agreement with the currently accepted value for the resting anaerobic heat of

Table IV.

No. of experiment ...	1	2	3	4*	5*	6*	7
Weight of muscle (g.)	1·32	1·41	1·52	1·11	1·28	0·72	1·50
Potentiometer reading (aerobic)	0·088	0·102	0·122	0·094	0·097	0·083	0·100
Potentiometer reading (anaerobic)	0·064	0·072	0·081	0·062	0·065	0·051	0·067
Heat production in g. cals. per hour (aerobic)	0·208	0·278	0·398	0·237	0·252	0·185	0·268
Heat production in g. cals. per hour (anaerobic)	0·110	0·139	0·176	0·103	0·113	0·070	0·120
Aerobic heat rate (g. cals. per hour per g.)	0·158	0·197	0·262	0·214	0·197	0·257	0·179
Anaerobic heat rate (g. cals. per hour per g.)	0·083	0·099	0·117	0·093	0·088	0·097	0·080

* In these experiments the muscle was rendered anaerobic by the addition of cyanide instead of the substitution of nitrogen for air. This procedure is seen to make no significant difference to the results.

normal frog's muscle; Hill for instance gives for this the figure 0·092 g. cals. per g. per hour.

Under aerobic conditions, there is not so much constancy in the actual figures obtained, varying as they do between 0·158 and 0·262 g. cals. per hour, with a mean value of 0·211. But the fact that this is more than twice as great as the anaerobic figure, and that in every instance the imposition of anaerobic conditions leads to a substantial fall in the resting heat-rate, furnishes evidence that in non-irritable muscle the oxidative mechanisms are not impaired.

Before the construction of the micro-calorimeter was completed, preliminary experiments along the same lines had been performed, using the Dewar flask technique which is described by Blaschko [1928] and employing large quantities of non-irritable muscle of the order of 20–30 g. The results obtained by this method, which are not incorporated in the present paper, were entirely in agreement with those here reported, in that the anaerobic heat-rate of non-irritable muscle was found to be the same as that of normal muscle, and that the aerobic heat-rate was usually about twice as large as the anaerobic. It was usually found that when this procedure was employed, the actual figures were if anything slightly lower than those yielded by the differential resistance thermometer.

D. The caloric quotient of non-irritable muscle.

The term "caloric quotient" is defined as the heat production (in g. cals.) associated with the anaerobic appearance of 1 g. of lactic acid. It is usually assumed to have a fairly constant value, in the neighbourhood of 390, and recent determinations under conditions of resting anaerobiosis and slight anaerobic fatigue by Meyerhof, McCullagh and Schulz [1930] did not result in any material alteration of this figure. Nevertheless, in the course of several unpublished experiments by one of us (D. R. McC.) it became apparent that this constancy was not invariably to be met with, and a number of instances were encountered in which the value obtained for the caloric quotient was very definitely higher than the accepted figure, although so far as was known

the muscle was in a perfectly normal condition. The fact that from time to time these high caloric quotients were obtained is of great interest from a biochemical standpoint, since the supposed constancy of this ratio is a weighty factor in the evidence for the belief that the only exothermic reaction of quantitative importance taking place in muscle is the transformation of carbohydrate to lactic acid. In cases where an abnormally high caloric quotient is observed, it is clear that there must be occurring other processes which are productive of heat and which do not culminate in the appearance of lactic acid.

The first experiments which were made with the object of studying the caloric quotient of non-irritable muscle were carried out by means of the Dewar flask calorimetric method, and the high figure obtained led us to repeat the observations, using the resistance thermometer, with a view to ascertaining whether the values for the caloric quotient were consistently high when the muscle was non-irritable. As will appear, this was found to be the case, and in no instance was a value lower than 600 obtained.

Technique.

It is desirable in experiments of this kind to avoid, so far as is possible, any injury to the muscle, as such injury leads to exaggerated lactic acid formation. It was customary therefore to use in each experiment the gastrocnemius muscles of two frogs; these muscles can be rapidly removed without damage to any of their fibres. After weighing, the gastrocnemii of the two right legs were placed in the tube V, together with the appropriate amount of water, while those of the left legs were placed in a similar tube with the same amount of water and were treated in an identical manner until the heat measurements were begun. The tubes were made anaerobic by the addition of $M/500$ cyanide and filling with nitrogen. Two or three hours were usually allowed for the apparatus to come to equilibrium, as it was found by experience that for the first hour or two after immersing muscles in water the heat production sometimes tended to be high and irregular, perhaps as a consequence of osmotic processes or perhaps on account of the utilisation of remaining traces of oxygen. At the time of commencing the readings, the tube containing the control muscles, which had hitherto been immersed in the bath, was rapidly cooled in a freezing mixture and its contents (muscle + water) were ground with the addition of a little sand and enough ice-cold 20 % trichloroacetic acid to bring the total volume to 10 cc. Lactic acid estimations were performed upon this, and thus the value for the commencement of the "heat-period" was known. (The assumption made here, that the lactic acid content of corresponding muscles from opposite limbs is identical, is invariably found to be a justifiable one.)

The experiments were allowed to continue for 20 to 24 hours. Preliminary tests had shown that at the end of such a period the muscles were in the same condition as when they were put into the apparatus; that is to say, normal

muscles remained irritable, and non-irritable ones remained non-irritable, but could be restored to irritability in oxygenated Ringer. Bacterial contamination was never encountered under these conditions.

At the expiration of the period, the contents of the calorimeter tube were treated exactly as the control muscles had been, and lactic acid estimations were performed. The apparatus was then calibrated in the usual manner, using 3 cc. of water in the tube V and passing a current through the heating coil.

Table V gives the results of a number of determinations made with normal gastrocnemii; a mean value of 375 is obtained for the caloric quotient. In Table VI are recorded similar experiments in which non-irritable muscle was substituted for normal muscle, and it will be seen that, while the values are considerably more variable, the average figure is much higher, *viz.* 740.

Table V. *Normal muscle.*

No. of experiment ...	1	2	3	4	5
Weight of muscle (g.)	1·20	1·38	1·09	1·23	1·06
Duration of experiment (hrs.)	24	20	20	24	20
Lactic acid before experiment (mg.)	3·9	3·5	4·3	4·5	3·2
Lactic acid after experiment (mg.)	10·9	10·3	9·6	12·4	8·5
Increase in lactic acid—A (mg.)	7·0	6·8	5·3	7·9	5·3
Potentiometer reading (volts)	0·063	0·070	0·059	0·071	0·060
Heat produced per hour (g. cals.)	0·107	0·131	0·093	0·135	0·096
Total heat produced—B (g. cals.)	2·57	2·62	1·86	3·24	1·92
Caloric quotient ($B/A \times 1000$)	367	385	351	410	362

Mean value of caloric quotient—375.

Table VI. *Non-irritable muscle.*

No. of experiment ...	1	2	3	4	5	6
Weight of muscle (g.)	1·31	1·02	1·22	1·05	1·28	1·17
Duration of experiment (hrs.)	20	22	22	20	20	24
Lactic acid before experiment (mg.)	4·4	3·0	3·7	3·9	4·8	3·0
Lactic acid after experiment (mg.)	7·5	5·1	8·2	6·5	8·8	6·2
Increase in lactic acid—A (mg.)	3·1	2·1	4·5	2·6	4·0	3·2
Potentiometer reading (volts)	0·067	0·056	0·068	0·060	0·069	0·059
Heat produced per hour (g. cals.)	0·120	0·084	0·124	0·096	0·128	0·093
Total heat produced—B (g. cals.)	2·40	1·85	2·73	1·92	2·56	2·23
Caloric quotient ($B/A \times 1000$)	774	881	607	739	640	697

Mean value of caloric quotient—740.

Since the resting anaerobic heat-rate is the same as in normal muscle, one is compelled to adopt the supposition that in non-irritable muscle the observed heat production is a result of changes other than lactic acid formation; changes which, owing to the suppression of the latter, assume an enhanced quantitative importance. It is not our intention at this stage to put forward any suggestions as to the nature of these processes. In view of these results it appears however that the value is considerably lessened of the supposed "constancy" of the caloric quotient and of the resting heat-rate as bases for the argument that lactic acid production is the main exothermic reaction taking place in muscle. Since the appearance of the work of Lundsgaard [1930] this argument has indeed become increasingly untenable, and there seems to be no escape

from the conclusion that the explanations, hitherto accepted as adequate, for the various thermal relations in muscular tissue will have to be considerably revised. Hartree, for instance [1931], has shown that poisoning by iodoacetic acid, while preventing lactic acid formation, has nevertheless no effect upon the heat distribution during contraction, and the same result has also been arrived at by Fischer [1931]. This may be compared with the fact reported in the present paper, that the resting heat-rate of non-irritable muscle is normal although less lactic acid is being formed. At the same time, the interference can scarcely be of the same, or even similar nature in the two cases, since stimulation of non-irritable muscle does not lead even to carbohydrate breakdown.

SUMMARY.

1. Upon electrical stimulation of "non-irritable" frog muscle, no lactic acid is formed and no carbohydrate disappears.
2. There is no heat production upon stimulation of non-irritable muscle.
3. The resting anaerobic heat-rate of non-irritable muscle is normal.
4. The oxidative mechanisms are intact.
5. The caloric quotient of non-irritable muscle is much higher than that of normal muscle, being of the order of 740 instead of 375.

Our thanks are due to Sir F. G. Hopkins for his continual interest in this research.

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CXXXVII. THE ERLENMEYER SYNTHESIS OF AMINO-ACIDS.

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Of the many methods available for the synthesis of α-amino-acids, that originated by Erlenmeyer [1893] has been most generally adopted by biochemists working in this field. For example, in more recent years, Ellinger and Flamand [1907], by its use, synthesised tryptophan, Pyman [1916] histidine, Barger and Ewins [1917] *Pr*-2-methyltryptophan, and Harington and Barger [1927], with a modified procedure, 3:5-diiodothyronine, the immediate precursor in the synthesis of thyroxine.

The main reasons for this choice are, firstly, that the starting materials are more or less readily available, and, secondly, that the benzoyl derivative of the amino-acid, obtainable at one stage of the synthesis, lends itself, as Fischer [1900] showed, to resolution into its optically active isomerides.

Nevertheless, the scheme, as Erlenmeyer himself pointed out, has certain weaknesses and these have been encountered repeatedly by later workers, *e.g.* Ellinger and Flamand, Pyman, Barger and Ewins.

The method as elaborated by Erlenmeyer consisted of four stages, *viz.*: (i) the formation of an oxazolone by the condensation of an aldehyde and hippuric acid; (ii) the hydrolysis of the oxazolone to an α-benzoylaminoacrylic acid; (iii) the reduction of the latter compound to the α-benzoylaminopropionic acid, and finally (iv) the hydrolysis of this compound to the α-amino-acid. Of these, stages (i) and (ii) proceed smoothly and with satisfactory yields. The same, however, cannot be said of stages (iii) and (iv).

The reduction as carried out by Erlenmeyer with the calculated quantity of sodium amalgam yielded only some 10–15 % of the theoretical amount of the saturated acid. This procedure was modified by Fischer who, using much larger quantities of sodium amalgam, obtained 80 % and 65 % yields respectively of benzoylphenylalanine and *N*-benzoyltyrosine. In other cases, however, the yields were persistently poor and moreover, the method has a further disadvantage. When the reduction has been completed, the unreduced acid remaining must invariably first be destroyed before the reduced compound can be isolated in a pure condition.

With regard to stage (iv), the hydrolysis, as carried out by these workers, takes a considerable time to accomplish and the yield of amino-acid is often

unsatisfactory. Thus Erlenmeyer found that benzoylphenylalanine, even after treatment in a sealed tube with concentrated hydrochloric acid for 16 hours at 140°, did not yield phenylalanine quantitatively, while Pyman, using Fischer's modification, reported that benzoylhistidine boiled with 60 times its weight of 20 % hydrochloric acid for 4 hours gave 70 % of the theoretical yield of histidine.

Harington and Barger [1927], in their synthesis of thyroxine on the lines of Erlenmeyer's method, were beset with yet another difficulty. To avoid removal of iodine by the alkaline reducing agent, they substituted for Erlenmeyer's sodium amalgam constant-boiling hydriodic acid and red phosphorus. On subjecting their benzoylaminoacrylic acid to this reducing reagent, they obtained, not the benzoylaminopropionic acid, but the α-amino-acid itself. Later Harington and McCartney [1927], modifying slightly this procedure by the introduction of acetic anhydride as a solvent, synthesised phenylalanine and tyrosine from the corresponding α-benzoylaminoacrylic acids or ethyl esters, whichever proved the more suitable.

Following the publication of these results of Harington and McCartney, Hoffmann-La Roche [1928, 1929] took out patents for the direct synthesis of thyroxine from the oxazolone, the reagent used being essentially that of Harington and McCartney. Apparently the oxazolone, like the benzoylaminoacrylic acid gives satisfactory yields of the amino-acid. Thus Erlenmeyer's original synthesis of an optically inactive α-amino-acid from the aldehyde had been reduced to a three-stage process [Harington and Barger, 1927], and to a two-stage process [Hoffmann-La Roche, 1928, 1929].

Nevertheless it will be seen that both these modifications, otherwise highly successful, suffer from one grave disadvantage. Reference has already been made to Fischer's use of the α-benzoylamino-acid for the purpose of resolution. Yet this is the very stage which is eliminated by the use of the acetic anhydride-hydriodic acid reagent, and thus we find Harington, in order to resolve his synthetic thyroxine (stage iv), under the necessity of doubling back to stage (iii) (he made formyldiiodothyronine), resolving and then hydrolysing the optically active isomerides into the respective amino-acids. It is apparent, therefore, that if the method of Harington and Barger could be so modified that the reduction took place without the simultaneous hydrolysis of the reduced compound, such a procedure would be a distinct advance.

Requiring certain amino-acid derivatives recently for metabolism experiments, and obtaining unsatisfactory yields with the sodium amalgam method of reduction, we turned our attention to the acetic anhydride-hydriodic acid method of Harington and McCartney. It occurred to us, in studying the method as described by these workers, that it might be modified in at least two directions, *e.g.*

(i) by variation of the amount of the hydriodic acid used, and

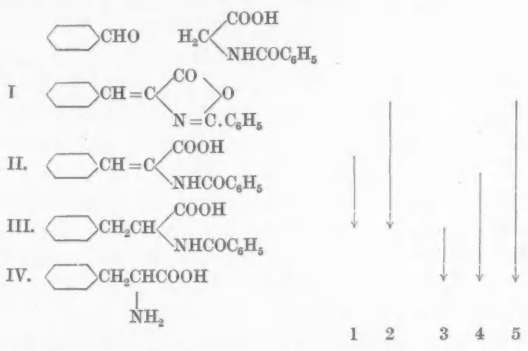
(ii) by the substitution of glacial acetic acid for acetic anhydride as the solvent.

Experimentation on these lines soon indicated that drastic reduction of the amount of hydriodic acid used led to the reduction of the unsaturated compound taking place without the loss of the benzoyl radical from the amino-group. For example, α-benzoylaminocinnamic acid gave benzoylphenylalanine, the product being obtained in excellent yield and in an easily purified form. The reaction was carried on for one hour, although it is probably complete in a shorter period of time. Treated with similar quantities of hydriodic acid, the oxazolone also yields the benzoylamino-acid, although, from results obtained, it would appear that the yield in this case is not quite so satisfactory as that obtained by the reduction of the acrylic acid.

From this point we were led to try the effect of increasing the amount of hydriodic acid, while still retaining acetic acid as the general solvent. It was now found that, should the hydriodic acid be present in sufficient quantity, the reduction was also accompanied by the removal of the benzoyl group, with the consequent production of the α-amino-acid. Thus α-benzoylamino-cinnamic acid under these conditions yields phenylalanine. Here again the yields are good and the product is easily purified. The reaction is complete in from 60–90 minutes, a period which, however, must be considerably lengthened if the hydriodic acid is required to effect the removal of other groups, *e.g.* methyl, as in the synthesis of tyrosine. With similar quantities of hydriodic acid, the oxazolone can be made to yield the α-amino-acid, whilst it also follows that the same method will remove the benzoyl group from the benzoyl-amino-acid.

It will thus be seen that the reaction can be so controlled that the one weakness of Harington and McCartney's procedure is eliminated. Moreover, the reagents involved are such that the synthesis can be carried out at a considerably reduced cost.

The results obtained may be summarised thus:



Reactions 1 and 2—by the use of minimum quantities of hydriodic acid.
Reactions 3, 4 and 5—by the use of much larger quantities of hydriodic acid.

Experimental.

Phenylalanine.

Benzoylphenylalanine. (*a*) From α-benzoylaminocinnamic acid. 10 g. of α-benzoylaminocinnamic acid, prepared according to the directions of Erlenmeyer [1893], were dissolved in 40 cc. of glacial acetic acid A.R., 1·6 cc. of hydriodic acid (sp. gr. 1·7) and 3 g. red phosphorus were added to the solution, and the resulting mixture was boiled under a reflux condenser for one hour. The hot solution was filtered through a glass filter, the separated phosphorus being afterwards washed with hot glacial acetic acid. The combined filtrate and washings were then evaporated to dryness *in vacuo*. Water was added and the evaporation to dryness repeated. The residue was taken up in the minimum quantity of hot alcohol, the solution boiled with a little charcoal and filtered. To the filtrate hot water was added until the solution became cloudy when it was set aside overnight. Practically colourless benzoylphenylalanine separated. A further crystallisation produced a product which melted at 184–5° (Fischer gives M.P. 186°). The yield was 82 % of the theory. (Found: N, 5·2. Calc.: N, 5·2 %.)

An alternative procedure, which, however, does not give quite such a good yield, is to pour the hot reaction mixture, after the removal of the red phosphorus, into a large excess of water. The crude benzoylphenylalanine which separates is purified as described above.

(*b*) From 4-benzylidene-2-phenyl-5-oxazolone. 10 g. of this oxazolone [Erlenmeyer, 1893] were boiled under a reflux condenser with a mixture of 70 cc. glacial acetic acid A.R., 1·6 cc. constant-boiling hydriodic acid and 3 g. red phosphorus for one hour. The reaction mixture was filtered hot and worked up in the manner already detailed. The benzoylphenylalanine, obtained in the first crystallisation from dilute alcohol, melted at 181°. The yield did not equal that obtained from the cinnamic acid, being only 72 % of the theoretical.

Phenylalanine. (*a*) From benzoylphenylalanine. 10 g. of benzoylphenylalanine, prepared by either of the above methods, were dissolved in 40 cc. glacial acetic acid A.R., and to the solution were added 40 cc. of hydriodic acid (sp. gr. 1·7) and a little red phosphorus, the resulting mixture being refluxed for 90 minutes. At the end of this time the hot mixture was filtered, the separated phosphorus washed with hot acetic acid, and the combined filtrate and washings were evaporated to dryness *in vacuo*. Water was added to the residue and the evaporation repeated. The residue was then dissolved in the minimum quantity of boiling water, the solution cooled under the tap, and extracted several times with ether to remove the benzoic acid and any unchanged benzoylphenylalanine. The aqueous solution was then concentrated to about 50 cc. On making it neutral to litmus by the addition of ammonia, the solution began to deposit almost colourless phenylalanine. After standing overnight, the phenylalanine was separated and recrystallised from boiling water. It

was thus obtained in the form of colourless plates. The yield of the analytically pure amino-acid was 5·3 g. or 80 % of theory.

(*b*) From α-benzoylaminocinnamic acid. 10 g. of α-benzoylaminocinnamic acid, 40 cc. of glacial acetic acid A.R., 40 cc. of constant-boiling hydriodic acid and 3 g. of red phosphorus were boiled together under a reflux condenser for 75 minutes. The coloured mixture which resulted was worked up in a precisely similar manner to that obtained in the preparation of phenylalanine from its benzoyl derivative. The yield was 82 % of the theoretical amount, and the product melted at 263–5° (Erlenmeyer gives 263°).

(*c*) From 4-benzylidene-2-phenyl-5-oxazolone. 10 g. of the oxazolone dissolved in 70 cc. of glacial acetic acid A.R. were boiled together with 50 cc. of constant-boiling hydriodic acid and 3 g. red phosphorus for 90 minutes. The subsequent treatment of the reaction mixture followed closely that already described. The yield of phenylalanine obtained amounted to some 68 % of that demanded by theory.

Tyrosine. 4-(p-*Methoxybenzylidene*)-*2-phenyl-5-oxazolone* was synthesised from anisaldehyde and hippuric acid in the usual way. 24 cc. of anisaldehyde, 60 g. of acetic anhydride, 40 g. of hippuric acid and 8 g. of freshly fused sodium acetate were well mixed in a conical flask and heated on the boiling water-bath for half an hour. The solid gradually went into solution and after 20 minutes the oxazolone began to separate; finally the whole became solid. The crude oxazolone was rubbed up with some 300 cc. of hot water and rapidly filtered, a process which was repeated twice. It was then dried in the steam-oven and finally crystallised from benzene, from which it separated in long needles. The product so obtained melted at 161–2°. It weighed 35 g. (62 % of theory). (Found: C, 72·7; H, 4·63; N, 4·8 %. Calc. for $C_{17}H_{13}O_3N$; C, 73·1; H, 4·67; N, 5·04 %.)

*α-Benzoylamino-*p-*methoxycinnamic acid.* The product of the last experiment was dissolved in the minimum quantity of hot alcohol, and to the solution was added 1½ times the theoretical equivalent of sodium hydroxide in the form of a 2 % aqueous solution. The mixture was kept hot and stirred on the boiling water-bath for 15 minutes. It was then cooled and neutralised (Congo paper) with dilute sulphuric acid. The almost colourless solid which separated was sucked as dry as possible on the Büchner funnel, washed several times with distilled water and finally crystallised from dilute alcohol. The yield was quantitative. The melting-point of the product was 230°. (Found: N, 4·6. Calc.: N, 4·7 %.)

*Benzoyl-*p-*methoxyphenylalanine.* (*a*) From α-benzoylamino-*p*-methoxycinnamic acid. A mixture of 10 g. of this acid, dissolved in 35 cc. of glacial acetic acid A.R., 1·4 cc. constant-boiling hydriodic acid and 2·5 g. of red phosphorus was boiled for one hour. The reaction mixture was filtered, and the separated phosphorus washed with hot acetic acid. The combined filtrate and washings were evaporated to dryness *in vacuo*. Water was added and the evaporation repeated. The residue was extracted several times with small

volumes of 10–15 % acetic acid, in which the tar present in the residue was insoluble. The filtrates were combined, boiled with a little charcoal, and filtered. Concentration *in vacuo* of the colourless solution so obtained led to the separation of the desired product. The yield was 65 % of the theory. The melting-point of the product was 173°, and this could not be raised by further recrystallisation from dilute acetic acid or alcohol. (Found: C, 68·1; H, 5·5; N, 4·62 %. Calc. for $C_{17}H_{17}O_4N$; C, 68·2; H, 5·7; N, 4·62 %.)

(*b*) From 4-(*p*-methoxybenzylidene)-2-phenyl-5-oxazolone. The method of preparation of the benzoyl-*p*-methoxyphenylalanine from the oxazolone was exactly similar to that in which the starting-point is the cinnamic acid, with the exception that the amount of glacial acetic acid employed was increased from 35 to 60 cc. The yield in this case was 57 % of the theory, while the product, after the first crystallisation, melted at 172°.

Tyrosine. From (*a*) benzoyl-*p*-methoxyphenylalanine; (*b*) α-benzoylamino-*p*-methoxycinnamic acid; (*c*) 4-(*p*-methoxybenzylidene)-2-phenyl-5-oxazolone.

The preparation of tyrosine from any one of these compounds followed closely that already described for the preparation of phenylalanine from its corresponding derivative. In order to effect the hydrolysis of the methoxy-group the time of reaction was increased from 1½ hours to 3½ hours. It was found best to treat the starting material with 10 times its weight of a mixture of equal volumes of glacial acetic acid A.R. and hydriodic acid (sp. gr. 1·7) together with 2–3 g. of red phosphorus. The yields obtained were (*a*) 75 %, (*b*) 63 % and (*c*) 55 % of those demanded by theory. The specimen of tyrosine prepared from benzoyl-*p*-methoxyphenylalanine darkened at 300° and melted in the neighbourhood of 316°. (Found: N, 7·7. Calc.: N, 7·75 %.)

One of us (W. R.) desires to take this opportunity to acknowledge his indebtedness to the Medical Research Council for a part-time grant.

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CXXXVIII. A PRELIMINARY NOTE ON THE INORGANIC SULPHATE CONTENT OF THE BLOOD WITH A METHOD FOR ITS DETERMINATION.

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The methods evolved for the determination of the inorganic sulphate content of the blood may be conveniently summarised as follows: (1) nephelometric, Denis [1921]; (2) volumetric, White [1923]; (3) gravimetric, Loeb and Benedict [1927]; and (4) colorimetric, Yoshimatsu [1926] and Hubbard [1927, 1930]. As the concentration of inorganic sulphate in the blood is very low, relatively large quantities of material are required for satisfactory gravimetric and volumetric analyses. It was therefore decided to investigate first the colorimetric method of Hubbard [1927, 1930]. In this method the blood-serum or blood-plasma is deproteinised with trichloroacetic acid, and the inorganic sulphate in the filtrate is then precipitated as benzidine sulphate in an acetone medium. The precipitated benzidine sulphate after solution in dilute hydrochloric acid is determined colorimetrically by the addition of hydrogen peroxide and ferric chloride, a yellow colour being produced. In their experiments with this method the present writers found that in the case of normal sera and plasma the concentration of inorganic sulphate was too low to produce a colour sufficiently intense to be of value for accurate comparison. There was, therefore, a need for a more sensitive colour reaction for benzidine.

Finally it was decided to modify the series of reactions used by Kahn and Leiboff [1928] for the determination of inorganic sulphates in urine, *viz.* diazotisation of the precipitated benzidine followed by coupling with phenol in alkaline solution. To intensify the colour the present writers have used thymol in place of phenol. The resultant colour is red.

Technique.

A solution containing 2·0071 g. benzidine hydrochloride in 500 cc. *N* HCl was prepared. Dilutions were then prepared of which 1 cc. was equivalent to 0·0025, 0·005, 0·01, 0·02, 0·04 and 0·08 mg. sulphur respectively. These dilutions were made with *N* HCl. To 2 cc. of each of these solutions 1 cc. of freshly prepared 0·1 % sodium nitrite was added. After shaking and allowing to stand 1 minute, 5 cc. of 15 % sodium hydroxide were added, and, after

thorough mixing, 5 cc. of 1 % thymol in 10 % sodium hydroxide were added to each. A red colour developed, very intense for the concentrations of benzidine chloride used. It was considered that this colour reaction would be much more suitable than the reaction used by Hubbard [1927, 1930].

Wakefield's [1929, 1] lowest standard was a dilution equivalent to 0·005 mg. sulphur, but it was found that a standard half this strength was necessary for the determination of the inorganic sulphate content of normal blood and serum. The colour obtained by coupling thymol with diazotised benzidine reached a maximum in 15 minutes and did not fade for 1 hour. The colour production was practically proportional to the amount of benzidine over this range, but in all estimations standards of varying benzidine content were prepared. In determining the inorganic sulphate content of blood, serum and plasma, all glass apparatus must be scrupulously clean and where potassium oxalate is used to prevent clotting, it must be shown to be free of all traces of sulphate.

The following technique was used for the determination of the inorganic sulphate content of blood, serum and plasma.

Reagents.

1. Acetone.
2. 20 % trichloroacetic acid in distilled water. Commercial trichloroacetic acid is purified by distillation with benzidine.
3. 0·5 % benzidine in acetone. This should be prepared fresh each day.
4. Benzidine hydrochloride standard solution.

2·0071 g. of benzidine hydrochloride are dissolved in 500 cc. *N* HCl. 1 cc. of this solution is equivalent to 0·5 mg. sulphur and is used as a stock solution. More dilute solutions for use as standards for the determination of inorganic sulphate in blood, being equivalent per cc. to 0·0025 mg., 0·005 mg., 0·01 mg., 0·02 mg., 0·04 mg. and 0·08 mg. sulphur, are prepared by diluting the stock solution with *N* HCl.

5. 0·1 % sodium nitrite solution—prepared fresh each day.
6. 15 % sodium hydroxide solution.
7. 1 % thymol in 10 % sodium hydroxide solution.

Method.

To 2 cc. of blood, serum or plasma are added 6 cc. of water and 2 cc. of 20 % trichloroacetic acid solution, and the whole is well shaken. This mixture is then either centrifuged or filtered. Most brands of filter-paper contain traces of sulphate which vitiate the results. This sulphate is insoluble in water but soluble in dilute acid solution, so that before use all filter-papers should be washed first with dilute acid, then with distilled water and finally dried in an oven. Into a 15 cc. centrifuge tube 2·5 cc. of the filtrate are measured, followed by 5 cc. of 0·5 % benzidine in acetone, and well mixed. After standing half an hour the benzidine sulphate is centrifuged and the supernatant fluid removed. The precipitate of benzidine sulphate in the centrifuge tube is washed

twice with acetone followed by centrifuging. Finally the centrifuge tube is inverted over a sheet of filter-paper until it is dry. The benzidine sulphate in the centrifuge tube is dissolved in 1 cc. of *N* HCl, with heat if necessary. After cooling, 0·5 cc. of 0·1 % sodium nitrite is added followed one minute later by 2·5 cc. of 15 % sodium hydroxide. After thorough mixing, 2·5 cc. of the alkaline thymol solution are added and after standing 15 minutes the coloured solution is compared with a suitable standard prepared at the same time. The standard is prepared as follows. 2 cc. of one of the dilute standard solutions are measured into a test-tube followed by 1 cc. of nitrite solution. After standing 1 minute, 5 cc. of 15 % sodium hydroxide solution are added, the whole is well shaken, and then mixed with 5 cc. of alkaline thymol solution. For normal blood it is only necessary to put up the lowest standard (1 cc. ≡ 0·0025 mg. S), but as a rule, to obtain standards approximating to the unknown, it is necessary to put up the whole series of standards.

As a preliminary, known amounts of ammonium sulphate were analysed in order to determine if the sulphate could be measured quantitatively by this method. The concentrations used were in the region of those sometimes found in plasma and serum, although higher than normal. These determinations were carried out under exactly the same conditions as in carrying out a determination with plasma and serum. From the results which are shown in Table I it will be seen that sulphate content could be quantitatively determined by this method with an error of less than 3 %.

Table I. *Determination of sulphate in standard solutions of ammonium sulphate.*

	mg. S per 100 cc. taken	mg. S per 100 cc. determined
1	0·91	0·90
2	1·83	1·79
3	0·91	0·93

Table II shows that added ammonium sulphate could be determined within an error of 5 %.

Table II.

	Inorganic sulphate content of blood-serum	Sulphate added	Total sulphate found	Sulphate recovered
1	1·55	1·14	2·67	1·12
2	1·55	2·28	3·95	2·40
3	1·55	4·56	6·22	4·67

The possible interference of phosphates and chlorides and of potassium oxalate, when plasma is used, must be taken into consideration. It was found, however, that 20 mg. P, 1 g. NaCl and 1 g. $(COOK)_2$ respectively per 100 cc. of blood, serum and plasma produced no appreciable effect on the determinations of sulphate.

In this work it was considered most convenient to express the sulphate content of blood in terms of S, to preserve the similarity with phosphorus and nitrogen.

The majority of workers have used serum or plasma in preference to whole blood for the determination of inorganic sulphate. Reed and Denis [1927], using a nephelometric method, state that in human blood the inorganic sulphate is found exclusively in the plasma. To test this point three different batches of normal oxalated blood were centrifuged and the cells washed twice with sulphate-free 0·85 % NaCl. 2 cc. of the corpuscular mass were mixed with 6 cc. of distilled water and then 2 cc. of 20 % trichloroacetic acid solution were added. After filtration the sulphate in the filtrate was estimated. No measurable coloration resulted. In order to make sure that the inorganic S had not dialysed through the red cell membrane the inorganic S contents of the whole blood and also of the plasma of nine different bloods were determined. Unfortunately haematocrit readings were not made but, averaging the values and assuming a cellular volume of 40 % of the whole blood, the inorganic content of the cells appears to be about one-fifth that of an equivalent volume of plasma.

Denis [1921] and Loeb and Benedict [1927] state that the retention of inorganic sulphate roughly parallels the retention of non-protein nitrogen, but Wakefield [1929, 2] states that there is no relation. It was decided to investigate this question. As a preliminary, the inorganic sulphate content of the sera of a number of normal healthy human subjects was determined. The results are shown in Table III. According to Wakefield [1929, 2] hospital patients suffering from diseases other than cardiac or renal are generally unsuitable for determining the normal range, since in many cases an increase in inorganic sulphate is observed even when there are no signs of renal inefficiency. It is extremely probable that in view of the extensive catabolic phenomena [Cuthbertson 1930, 1931], which result from injury, certain changes will occur in the blood, so that a great many hospital patients must therefore be excluded from any normal survey.

With four exceptions all the cases herein described were healthy men and women between the ages of 20 and 40 years.

Table III. *Sulphur content of normal plasma.*

	Case	Mg. S per 100 cc.	Case	Mg. S per 100 cc.	
Aet. 6 years	1	0·4	11	0·3	
13 „	2	0·4	12	0·1	
19 „	3	0·3	13	0·2	
	4	0·1	14	0·45	
	5	0·2	15	0·3	
	6	0·4	16	0·5	
	7	0·4	17	0·4	
	8	0·2	18	0·5	
	9	0·1	19	0·2	
	10	0·3	20	0·5	
			21	0·45	aet. 56

The inorganic sulphur content of the plasma or serum of normal fasting men and women appears to vary from 0·1 to 0·5 mg. per 100 cc. Other workers have obtained the following normal values expressed as mg. S per 100 cc.:

Denis [1921] plasma, 0·2–0·4 mg./100 cc.; Loeb and Benedict [1927] serum, 0·7–1·6 mg./100 cc.; Wakefield [1929] serum, 0·2–0·9 mg./100 cc.

The higher normal values quoted by Loeb and Benedict [1927] may perhaps be ascribed to the error of gravimetric estimation of amounts of barium sulphate of the order of 0·2 to 0·7 mg.

A few analyses of hospital cases are given in Table IV. They do not include any cases of obvious renal disease among their number.

Table IV.

Case	Disease	Mg. S per 100 cc.
22	Osteomalacia	0·5
23	Rheumatoid arthritis	0·2
24	Osteomalacia	0·9
25	Osteitis deformans	0·9
26	Fractured skull	0·7
27	Diabetes mellitus	0·9
28	Diabetes mellitus	0·7
29	Normal pregnancy	0·3

Forty cases of nephritis and cardio-renal disease have been investigated to determine what relation, if any, exists between the urea and inorganic sulphate content of the blood.

The urea content of the blood was determined on the whole fluid, but since this compound is contained in equal amount in plasma and red cells, the resultant values can be legitimately compared with the inorganic sulphate content of the plasma or serum.

Without considering particularly the various forms of nephritis and cardio-renal disease, but arranging the various cases in order of increasing retention of urea-nitrogen, it will be found that in general there is a coincident increase in the sulphur content and that on dividing the 40 cases into four arbitrary groups of 10 and determining the S : N ratios the values are fairly constant, 1 : 14·2, 1 : 14·7, 1 : 14·1 and 1 : 19·1. In the last group the sulphur increment lags behind the increment in urea-nitrogen (see Table V and Fig. 1).

Urea has been chosen for comparison with the inorganic sulphate, since its metabolism is in general parallel. It must be emphasised, however, that these are only average values and that the individual fluctuations are numerous and considerable. It is interesting to consider, however, that these ratios are of practically the same order as the S : N ratio of muscle, while those of the blood of normal cases are much lower (*ca.* 1 : 50).

If, however, there be deducted from these total retention values, the presumed normal content of urea-nitrogen and inorganic sulphate (15 mg. urea-N; 0·3 mg. inorganic S; S : N ratio 1 : 50), values are obtained which indicate that relative to urea-nitrogen, inorganic sulphate is retained in greater amount in the lower grades of retention, and that as the retention values increase, nitrogen relative to sulphur is retained in increasingly greater amount (S : N ratios 1 : 2·2, 1 : 8·1, 1 : 10·7 and 1 : 17·4).

From these data it appears that the measurement of the inorganic sulphate

content of the plasma or serum may prove to be of more service in the detection of mild degrees of renal impairment than the determination of the urea or non-protein nitrogen level.

Table V. *N mg. per 100 cc. whole blood; inorg. S mg. per 100 cc. plasma.*

Case	N.P.N.	Urea-N	Inorg. S	Case	N.P.N.	Urea-N	Inorg. S
30	32	13	0·3	40	58	22	1·8
31	30	15	0·6	41	52	23	2·6
32	35	15	0·8	42	40	23	0·6
33	56	15	2·3	43	63	23	1·2
34	41	16	1·6	44	63	27	1·6
35	33	17	1·0	45	70	28	0·9
36	43	18	1·8	46	80	29	4·0
37	39	20	0·4	47	60	32	1·4
38	41	20	0·9	48	59	33	2·9
39	52	22	2·3	49	80	36	2·3
Average		17	1·20			28	1·9
N : S		14·2 : 1				14·7 : 1	

Case	N.P.N.	Urea-N	Inorg. S	Case	N.P.N.	Urea-N	Inorg. S
50	56	37	2·9	60	109	60	5·1
51	87	39	3·1	61	150	70	6·0
52	69	40	3·4	62	115	83	6·1
53	64	42	2·4	63	106	88	4·2
54	79	48	4·8	64	137	100	4·2
55	71	50	2·9	65	132	111	4·9
56	82	50	2·4	66	174	120	5·7
57	112	50	2·4	67	228	132	6·3
58	100	50	2·4	68	209	158	8·9
59	109	55	5·2	69	236	177	6·1
Average		46	3·2			110	5·75
N : S		14·1 : 1				19·1 : 1	

Correlation coefficient between urea-N and inorg. S

$M_{\text{urea-N}} = 50{\cdot}25 \pm 4{\cdot}309$; $M_{\text{inorg. S}} = 3{\cdot}01 \pm 0{\cdot}212$

$\sigma_{\text{urea-N}} = 40{\cdot}405 \pm 3{\cdot}047$; $\sigma_{\text{inorg. S}} = 1{\cdot}993 \pm 0{\cdot}1503$

$r_{\text{urea-N, inorg. S}} = +0{\cdot}85 \pm 0{\cdot}028$

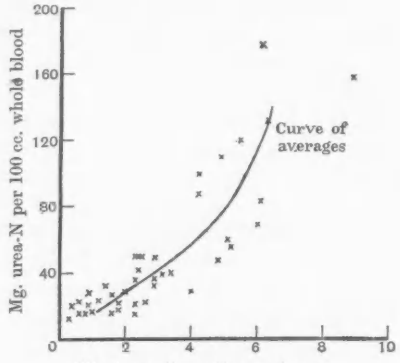

Fig. 1.

SUMMARY.

1. A method has been described for the determination of the inorganic sulphur content of the whole blood, plasma or serum. The method requires 2 cc. fluid.

2. Concentrations of phosphate, chloride and oxalate in the blood of the respective orders—20 mg. P, 1000 mg. NaCl and 1 g. $K_2C_2O_4$ per 100 cc.—do not affect the reaction.

3. There is very little inorganic sulphur in the cellular constituents of the blood.

4. The normal content for healthy adult men and women varies from 0·1 to 0·5 mg. inorganic S per 100 cc. plasma.

5. In cases of nephritis and cardio-renal disease there is on the average an increased retention of inorganic sulphur coincident with the increased retention of urea- and non-protein-nitrogen. Relative to nitrogen, inorganic sulphur is retained in proportionately greater amount in the minor degrees of retention than in the more severe types where relative to nitrogen it is less prominent.

The coefficient of correlation as will be seen from the calculation at the foot of Table V is $r_{\text{urea-N, inorg. S}} = + 0{\cdot}85 \pm 0{\cdot}028$.

In conclusion we wish to express out thanks to Dr G. M. Wishart for help with the statistical survey.

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CXXXIX. THE SENSITISATION OF RED BLOOD CORPUSCLES IN HAEMOLYSIS.

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(*Received July 2nd, 1931.*)

The sensitisation of red blood cells by means of a specific amboceptor and their lysis by serum complement have been known to exist for a long time, but in recent years some cases have been found where this sensitisation can be produced by chemicals, though it cannot be definitely stated whether the analogy is only superficial or not. Sachs [1908] noticed a long time ago that when washed red blood cells were treated with low concentrations of soaps and then with normal serum, a marked acceleration of haemolysis took place. No attention was apparently paid to this type of work till Ponder [1922, 1923, 1924] described certain experiments on the accelerating effect on haemolysis of normal serum and other proteins when added to a suspension of washed cells previously treated with a bile salt. This observation enabled him to give a logical explanation of the percentage haemolysis curve for taurocholate haemolysis, where a great acceleration of haemolysis takes place after the haemolytic reaction has proceeded for some time—a fact due to the effect of liberated haemoglobin. Ponder also found that histamine could replace normal serum to a certain extent in this acceleration process. Recently he [1928] has repeated the work of Browning and Mackie [1914] and of Mackie [1919] on haemolysis by brilliant green and its acceleration by serum. In several previous papers [Sen *et al.* 1929, 1931] the present author studied this acceleration of haemolysis by different substances, and the view was expressed that the acceleration of haemolysis might be due to a sudden variation of the p_H at the cell surface which then induced further physico-chemical changes. The object of this paper is to give more evidence on this point. The present position of the problem, of great importance for the theory of haemolytic reactions in general, may be stated as follows. Since red blood cells can be sensitised by treatment with bile salts, soaps or dyestuffs like brilliant green, and then show a markedly lower resistance to normal serum, the question arises as to whether the action of normal serum is a continuation of the process started by the sensitising agent or not. It is known that all these sensitising agents are more or less adsorbed by the cells and are in themselves haemolytic at higher concentrations. It is be to assumed therefore that the adsorption of these substances, though not sufficient to cause lysis within the time given on account of the low concentration used, does actually change the cell surface

in such a way that some of the components of normal serum can act haemolytically. In studying the effect of normal serum on sensitised corpuscles, we can consider separately the effect of different major constituents of the serum—namely the proteins and the salts. From the work of Sachs, Ponder and others, it appears that the protein fraction is effective. Evidence is given in this paper that under many conditions the saline fraction of the serum solutions at the same p_H level is equally effective as the normal serum itself, and often the acceleration is determined by the amount of OH^- ions added.

Experimental.

In all the experiments recorded below, sheep's erythrocytes have been used after being defibrinated and washed four times with normal saline. The concentration is expressed in terms of cc. of corpuscles per 100 cc. of saline suspension. The sensitising substance (bile salt) was also freshly prepared in saline. The time of complete haemolysis was obtained by a colorimetric comparison with a standard.

As is well known, a haemolytic experiment in presence of normal serum can be made in one of the following three ways: (1) the serum may be added to the mixture of corpuscles and bile salt (sensitiser); (2) bile salt may be added to the mixture of corpuscles and serum; and (3) a mixture of bile salt and serum may be added to the corpuscles. The effect of serum in (2) always gives an inhibition of haemolysis, in (3) there may be inhibition or acceleration, but usually inhibition, and in (1) there also may be either inhibition or acceleration depending upon several variables. All these effects due to normal serum have been imitated by using a bicarbonate-Ringer solution instead of serum. As however we are more concerned with the procedure in (1), which is analogous to the amboceptor-complement reaction, we shall only deal with this. An example of the effect of serum obtained according to (1) and (3) may be given here in order to serve as a model for the presentation of results with substances other than serum. In Table I the concentration of cells is 1 % and of taurocholate 0·3 %. In each experiment 0·5 cc. cells and 0·5 cc. taurocholate in a total volume of 5 cc. have been used.

Table I.

(1)	Time of haemolysis of the control	3 mins. 21 secs.
(2)	0·5 cc. serum + taurocholate added to cells	No haemolysis in 2 hours
(3)	0·5 cc. serum added to cells + taurocholate ½ min. after adding the taurocholate to the cells	1 min. 4 secs.

This accelerating effect was analysed further by varying the amount of serum added, determining the effect of the time interval during which sensitisation is allowed to take place, the concentration of the corpuscles and of the sensitising agent, *etc.*, and it was found that all these factors had some effect on the observed acceleration. Under certain conditions, using the same technique, an inhibition instead of acceleration could be observed. Thus Fig. 1,

in which the experiments given in Table I (3) have been repeated with varying amounts of serum added to a sensitised corpuscle mixture after ½ minute,

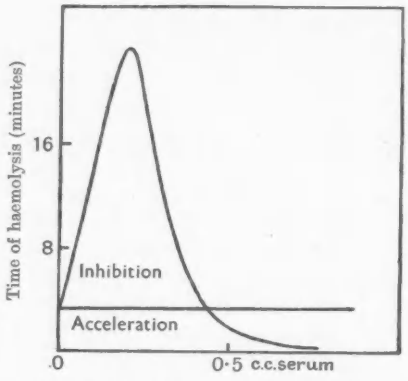

Fig. 1. Effect of serum on sensitised cells.

shows a well-marked inhibition and acceleration of haemolysis depending upon the amount of serum added. It was also found that the amount of serum necessary to induce the acceleration of haemolysis was much less when the time-interval given for sensitisation was greater. Both these effects of serum have been imitated by various substances with one common property, namely that of generating OH^- ions in solution. The experimental work falls mainly into three groups: (*a*) determination of inhibition-acceleration curves in presence of substances which give OH^- ions in solution, such as sodium hydroxide, ammonia, different amines and alkaline buffer systems; (*b*) measurements of the p_H of solutions after the addition of different amounts of these substances to sensitised cells in order to find out if there is any correlation between the change in the p_H and the observed inhibition and acceleration of haemolysis; and (*c*) the effect of time of sensitisation on the action produced by these different alkaline substances.

The general results are summarised as follows.

(*a*) If the concentration of the corpuscles and the amount of the sensitising agent (taurocholate in this case) are rightly chosen, and a suitable interval is given after which OH^- ions are added, it is easy to obtain both inhibition and acceleration with all the substances mentioned above. The concentrations can be easily adjusted by several trials. Some typical curves are shown in Figs. 2 and 3. In Fig. 2, the final cell concentration is 0·2 %, taurocholate approximately 0·01 %, and the time-interval after which ammonia is added is ½ minute. In Fig. 3 and curve *B* of Fig. 6, the cell concentration is 0·1 %, taurocholate 0·03 % and the time-interval after which dipotassium hydrogen phosphate or Ringer-bicarbonate has been added is ½ minute. It will be seen that the nature of the results is exactly the same as observed in the case of serum.

(*b*) Having determined the type of curves obtained by varying the amounts of the alkaline substances, it was decided to find out the relation, if any,

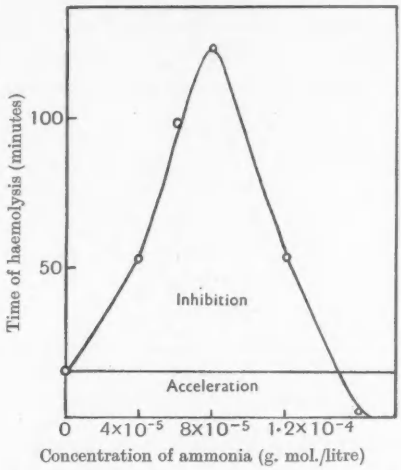

Fig. 2. Effect of ammonia on sensitised cells.

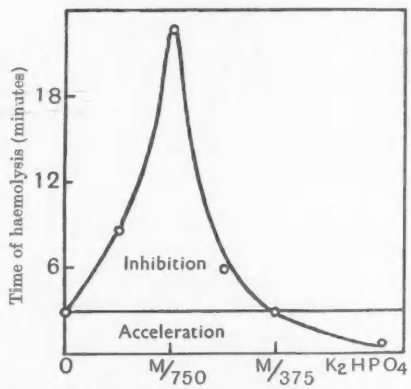

Fig. 3. Effect of dipotassium hydrogen phosphate on sensitised cells.

between the p_H change and the inhibition and acceleration of haemolysis. For this reason, the p_H of the final solutions after addition of normal serum or any of the above-mentioned substances was measured with a quinhydrone electrode. In these measurements, the final concentration of the cells was 0·1 %, and it was found that, with the concentration of haemoglobin derived from the cells, the error due to a drift in the potential could be neglected. The following solutions were used. Normal serum p_H 8·0, dipotassium hydrogen phosphate

$M/15$, Ringer-bicarbonate p_H 8·5, and 1 % gelatin in saline made alkaline with NaOH, p_H 8·5. Small amounts of these solutions were used in the actual experiments in order to vary the p_H, which was finally determined electrometrically. The corpuscle suspension had an initial p_H of 6·9 and the sensitised suspension had also almost the same p_H. In Figs. 4 and 5, two typical results

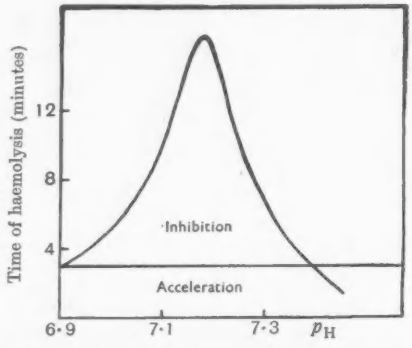

Fig. 4. Effect of p_H change on sensitised cells. Serum as accelerator.

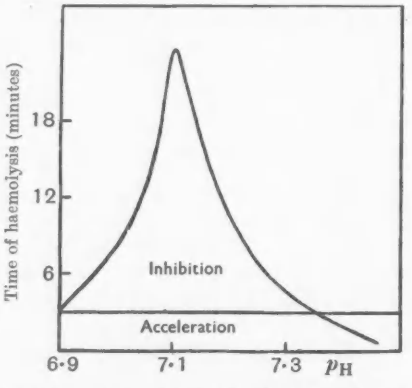

Fig. 5. Effect of p_H change on sensitised cells. Potassium phosphate as accelerator.

with serum and phosphate are shown. The curves show clearly the range of p_H over which an inhibition of haemolysis is observed. This fact, that acceleration of haemolysis only results when the p_H change is sufficiently high, seems to indicate that the lytic activity of normal serum on sensitised corpuscles is due to its alkali content. Further experiments however show that the limit of p_H beyond which the acceleration is possible is not a constant one, and the phenomenon is more complex than was thought at first.

(*c*) It has already been stated that the time-interval after which the serum or the alkaline substances are added has a definite effect on the inhibition or

acceleration of haemolysis. A typical result obtained with Ringer-bicarbonate is shown in Fig. 6. The time of complete haemolysis has been determined after

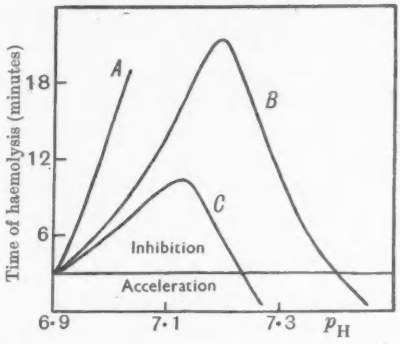

Fig. 6. Ringer-bicarbonate as accelerator. *A*. Sensitisation time, 15 secs. *B*. Sensitisation time, 30 secs. *C*. Sensitisation time, 1 minute.

allowing variable periods to elapse before the bicarbonate solution was added to cells in the course of sensitisation by taurocholate. The p_H of the solutions was measured after the completion of the experiment. The following points in this figure are to be noted. When the time of sensitisation is 15 seconds (curve *A*), the addition of bicarbonate sufficient to raise the p_H to 7·6 does not accelerate the haemolysis, but retards it. When the time of sensitisation is 30 seconds (curve *B*), an acceleration is obtained as soon as the p_H exceeds 7·4. With a time-interval of 1 minute (curve *C*), the p_H limit comes down to 7·25, and with a time interval of 2 minutes (not shown in the figure) there is practically no inhibition, and the p_H range comes down to 7·05–7·1. It is to be noted here that this last time-interval is very close to the time necessary (3 minutes) for the haemolysis of the control. I have not been able to get any acceleration below p_H 7·0 under these experimental conditions, but it cannot be stated from these results that the acceleration is determined purely by the p_H change of the medium, as the magnitude of the change is sometimes very small. It is very likely that a large change in p_H is one of the factors by which we can obtain an acceleration of haemolysis, and it seems that during the sensitisation process the surface of the cells undergoes some irreversible changes making its stability conditions highly susceptible to p_H changes.

Attention may now be drawn to the effect of H^+ ions on sensitised cells. Thus it has been found that under certain conditions, with bile salt as sensitiser, a change of p_H from 6·9 to 6·4 made by the addition of acetate buffer would give an acceleration of haemolysis. No inhibition of haemolysis by acids could however be observed. It appears therefore that in bile salt sensitisation, the surface or some particular part of the surface of the corpuscle becomes so much changed, that a variation of p_H in either direction would cause a lysis

of the cells. This effect of acid is very marked when cells are sensitised by saponin, but with normal serum no sensitisation by saponin can be observed.

In conclusion two points of interest in connection with the acceleration of haemolysis in presence of normal serum may be mentioned. It has already been stated that though a mixture of normal serum and bile salt usually shows an inhibition of haemolysis, yet under some conditions this mixture might have a greatly increased haemolytic power. This fact of course is not solely dependent on the proteins, as a similar effect has been observed with a mixture of bile salt and either ammonia or ethylamine. The conditions under which this greatly increased haemolytic efficiency is observed have not been studied thoroughly, but it appears that there are at least three variables in this, namely, concentration of the cells, concentration of the bile salt and the concentration of serum or the alkaline substance used as the inhibitors. The second point of interest is the the fact that though serum and certain other substances will haemolyse a sensitised suspension and some variables in this reaction have been mentioned, there is another variable, namely the condition of the cells themselves, which profoundly affects the results. This point has been previously emphasised by Ponder.

Summary.

1. In a study of the effect of normal serum on corpuscles sensitised by bile salts, it is found that both inhibition and acceleration of haemolysis can be observed under suitable conditions.

2. It is found that this effect of normal serum is not solely due to the proteins, but the alkali-content of the serum must have some effect.

3. This effect of serum has been imitated by many substances which yield hydroxyl ions in solution.

4. Erythrocytes can be highly sensitised towards acids by previous treatment with bile salt or saponin.

5. The acceleration of haemolysis of corpuscles sensitised by bile salts due to the addition of serum or other acid or alkaline substances cannot be considered to be entirely dependent on the p_H change of the solution. The time allowed for the sensitisation to take place has a great influence on the effect of p_H change and acceleration of haemolysis.

6. Normal serum acts both as a haemolytic agent and an anti-haemolyte depending on the conditions of the experiment, and it appears that the haemolysis of sensitised cells by serum will have to be considered as a continuation of the action started by the sensitising substance.

I am much indebted to Sir F. G. Hopkins for his kind interest in this work.

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CXL. THE OXIDATION OF OLEIC ACID BY MEANS OF HYDROGEN PEROXIDE WITH AND WITHOUT THE ADDITION OF COPPER SULPHATE: A POSSIBLE ANALOGY WITH ITS OXIDATION *IN VIVO*.

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(*Received July 3rd, 1931.*)

The discussion of the method of biological oxidation of the fatty acids has hitherto been almost entirely confined to the saturated acids, in spite of the fact that the acid which occurs in greater proportion than any other in the large majority of fats is the unsaturated oleic acid. Such facts as are available point to the conclusion that where oxidation is taking place rapidly the fat contains a relatively large proportion of unsaturated acid. Lewkowitsch quotes figures, for instance, showing that the fat of animals in the wild state is more unsaturated than that of the same species reared in domesticity: the iodine value of the fat of the wild rabbit is 101 as compared with 64 for the fat of a tame rabbit, and similar differences hold for the goose and the hog. Although it has been established [Ellis and Zeller, 1930] that carbohydrate feeding leads to a diminution of the unsaturated acids in the storage fat, it is uncertain whether variations in diet would account for the whole of the above differences, and it is possible that where the animal is leading a more active life a higher proportion of unsaturated acids is available. In the fat of the lower organisms the proportion of unsaturated acid is greater when they are grown at a low temperature and diminishes as the temperature at which they are grown is raised [Terroine, *et al.* 1927; Pearson and Raper, 1927]. Blood-plasma contains a high proportion of unsaturated acids, a large proportion of which are more unsaturated than oleic acid [Bloor, 1924]. In the liver oils the proportion of saturated acids is comparatively low. Bloor [1926, 1927] has shown that in the fat extracted from heart muscle and from voluntary muscle it is the unsaturated acids which predominate, the proportion being greater than in storage fat. The oxidation of the unsaturated acids in the body appears to be a normal process and it is generally held, largely owing to the work of Leathes and his colleagues, that desaturation of the fatty acids precedes oxidation. Nothing, however, is known of the stages by which oleic acid breaks down in the body and the theory of β-oxidation, which has found most acceptance as the probable method by which the saturated acids containing normal chains

are oxidised, does not necessarily hold good in the case of the unsaturated acids, since here oxidation must first take place at the ethylenic linkage.

In the laboratory the oxidation of oleic acid by the action of potassium permanganate has been carefully investigated. Saytzeff [1886] showed that dihydroxystearic acid was produced when oleic acid was treated with a dilute alkaline solution of permanganate. Lapworth and Mottram [1925], working with the same reagent, found that, if the permanganate solution were sufficiently dilute and the temperature between 0° and 10°, the conversion of oleic acid to 9 : 10-dihydroxystearic acid was almost quantitative.

By the further action of permanganate Edmed [1898] obtained nonoic, azelaic and oxalic acids, and previously Spiridonoff [1887] using the same reagent had isolated sebacic, suberic and caprylic acids. Lapworth and Mottram [1925] reconciled these results and showed that the oxidation of the dihydroxystearic acid first formed can take place in two ways according to the following equations:

(1) $CH_3.(CH_2)_6.CH_2.CH(OH).CH(OH).CH_2.(CH_2)_6.COOH + 3O =$
$CH_3.(CH_2)_6.CH_2.COOH + COOH.CH_2.(CH_2)_6.COOH + H_2O.$
pelargonic acid azelaic acid

(2) $CH_3(CH_2)_6.CH_2.CH(OH).CH(OH).CH_2.(CH_2)_6.COOH + 8O =$
$CH_3.(CH_2)_6.COOH + COOH.COOH + COOH.(CH_2)_6.COOH + 2H_2O.$
caprylic acid oxalic acid suberic acid

The latter reaction is favoured by a more dilute solution and a lower temperature.

It is certainly improbable that an unsaturated acid containing an ethylenic linkage in the 9.10-position would in the first instance undergo oxidation in the β-position. Such migrations of the double bond as are known to take place in the fatty acids are only brought about by violent chemical reagents often acting at high temperatures. A shift from the 9.10- to the 3.4-position, for instance, is brought about when oleic acid is treated with concentrated sulphuric acid, giving rise to stearolactone, and when oleic acid is fused with caustic potash [Edmed, 1898; Le Sueur, 1901] a change of the unsaturated linkage from the 9.10- to the 2.3-position must be involved, since palmitic and acetic acids are obtained. Although this conversion of oleic to palmitic acid is exceedingly interesting, it is difficult to regard a process brought about by fusion with caustic potash as affording grounds for believing that such a change is brought about by enzyme action at the body temperature. When laboratory experiments are used as a basis for suggesting analogies with the processes actually occurring in the body, it is usual to choose reactions which can be carried out at a comparatively low temperature by the aid of mild chemical reagents such as might conceivably be present in the body. Such a method is the oxidation with hydrogen peroxide studied by Dakin and other workers, although in the case of the higher fatty acids Dakin found it necessary to carry out the reaction at 95°. Even at this temperature only about 10 % of the original acid is attacked, but Dakin [1908, 1910] succeeded in isolating the

ketones derived from the β-hydroxy-derivatives of the fatty acids by the elimination of CO_2. He quoted these experiments as furnishing an instance of oxidation under conditions not too far removed from those obtaining in the body and advanced them in support of the view that the degradation of the saturated fatty acids takes place in the body by a repeated process of β-oxidation. However, if the β-keto-acids are formed in the body, all except β-ketobutyric acid must be immediately further broken down without the separation of the corresponding ketones, since the only fatty ketone known to occur in the body is acetone, and there is no evidence that the higher ketones would be readily attacked.

The possible conversion of the β-hydroxy-acids to $\alpha\beta$-unsaturated acids also lacks any experimental foundation.

Clutterbuck and Raper [1925] have shown that in the action of hydrogen peroxide on the saturated fatty acids, oxidation occurs in the γ- and δ-positions at the same time as in the β-position and to about an equal extent.

Hazura [1888] investigated the oxidation of oleic acid with hydrogen peroxide in the absence of an alkali at ordinary temperature but failed to detect any evidence of oxidation.

The present communication records the results of the oxidation of this acid under the experimental conditions used by Dakin and his colleagues and also by the method described by Battie and Smedley-MacLean [1929] in which, by the addition of a cupric salt as catalyst, oxidation proceeds much more readily and at a lower temperature.

The oxidation of oleic acid with hydrogen peroxide at 95°.

4 g. of oleic acid were used in each experiment, the oxidation being carried out in slightly alkaline solution and a sufficient quantity of the peroxide added to furnish four atoms of oxygen for each molecular proportion of oleic acid. The reaction mixture was heated at 95° for 3 to 3·5 hours. Acid was then added and the CO_2 blown off and absorbed by baryta, and the separation into various fractions carried out as described on p. 1260. After the carbonic acid had been removed, the insoluble oil floating on the surface was filtered off: this formed nearly 90 % by weight of the oleic acid originally taken.

The carbonic, acetic and formic acids produced account for less than 10 % of the carbon of the oleic acid originally present. The neutral volatile distillate gave a positive reaction with sodium nitroprusside, which was probably due to acetone since we succeeded in isolating a very small amount of crystals of a dinitrophenylhydrazone which melted at 121–123°. A specimen of acetone dinitrophenylhydrazone melted at 123–124°. A trace of acetone was, therefore, probably present. A small amount of acid (0·16 g.) was obtained from the aqueous reaction mixture by continuous extraction with ether. There was too little to be purified satisfactorily, but since it gave an iodoform reaction in the cold and a molecular weight of 151 by titration, it was evidently similar to the 4-keto-7-hydroxycaprylic acid isolated in much larger quantity in subsequent

experiments when copper had been added to the reaction mixture. We were unable to detect succinic acid in the small amount of material available but we can say with certainty that not more than a trace of this acid could have been present. A trace of an acid soluble in water and insoluble in ether appeared to be oxalic acid.

The main fraction of the oil was insoluble in water. It was neutralised with $N/2$ NaOH and the aqueous solution extracted with ether to remove any neutral substances present.

The neutral fraction was present only in small amount, the total weight being about 5 % of the oleic acid originally taken. It was separated into two parts by gently warming its alcoholic solution on a water-bath and adding to it at intervals small quantities of $N/2$ NaOH until the liquid remained just alkaline. After removing the alcohol the slightly alkaline solution was extracted with ether and a small amount of a neutral substance isolated. Four specimens of the fraction, which did not dissolve on warming with dilute alkali, were obtained in different experiments and were analysed with the following results:

	% C	% H	
(4)	75·70	11·90	
(3)	75·25	11·96	
(2)	74·71	11·65	
(1)	74·25	11·79	(least pure specimen, probably contaminated by lactone)

These figures agree best with the figures required by a diketoheptadecane $C_{17}H_{32}O_2$ (C, 76·12; H, 11·90 %). Attempts to prepare an oxime or dinitrophenylhydrazone were, however, unsuccessful and the nature of this substance has not been satisfactorily settled. The only possible product of β-oxidation would, however, be contained in this fraction, so that we can say that if under the conditions of this experiment β-oxidation does take place, it can occur only to a very slight extent and is certainly not the main path of the reaction.

Acidification of the solution produced by warming the neutral fraction with dilute alkali set free an oil with a pleasant characteristic odour, which was left in a desiccator until of constant weight and then analysed. It contained C, 69·70, 69·79; H, 10·49, 10·71 %, thus agreeing with the figures required by the lactone of a ketodihydroxystearic acid, $C_{18}H_{32}O_4$, C, 69·23; H, 10·25 %. Oxidation had, therefore, probably taken place in the γ- or δ-position as well as at the ethylenic linkage.

The acid fraction was extracted by means of light petroleum from the aqueous solution after it had been acidified; a small fraction remained insoluble in light petroleum from which crystals were obtained melting at 130°; these were identified as dihydroxystearic acid by a determination of a mixed melting-point. The composition of the oil which dissolved varied somewhat in different experiments; its iodine value varied between 48 and 58, so that if it is justifiable to regard this as due to the presence of oleic acid, from 53 to 65 % of the fraction consisted of unchanged oleic acid, *i.e.* from 40 to 50 % of the starting material had not undergone any oxidation. The presence of

oleic acid in the fraction was confirmed by oxidation with a dilute solution of potassium permanganate at 5° and the resulting dihydroxystearic acid was identified by analysis and determination of melting-point.

It was found that a separation of the fraction might be made by means of its silver salts. The oil was dissolved in alcohol, neutralised, the silver salt precipitated in boiling solution, filtered off and well washed with hot water. The insoluble silver salt was then repeatedly heated with boiling water, and the washings added to the solution of the soluble silver salt, which was then acidified and extracted with ether. The oil obtained in two separate experiments contained C, (1) 64·69, 64·65; (2) 63·01: H, (1) 9·70, 9·78; (2) 9·29 %. Mol. wt. by titration = (1) 188; (2) 198. Iodine value = 0. Calculated for ketodecoic acid, $C_{10}H_{18}O_3$, C, 64·52; H, 9·68 %. Mol. wt. = 186: for ketononoic acid C, 62·77; H, 9·42 %. Mol. wt. = 172.

A silver mirror was deposited on the sides of the tube when a solution of the sodium salt was warmed with an ammoniacal solution of silver nitrate. When heated with silver oxide and water, CO_2 was evolved and a volatile acid with a slightly goaty smell deposited on the sides of the tube. An α-ketoic acid is therefore present, a derivative of either nonoic or decoic acid. It was thought possible that the semialdehyde of azelaic acid might be present in this fraction, but no evidence was obtained of the presence of azelaic acid on oxidation.

The acid obtained from the insoluble silver salt contained from 71 to 75 % C and from 10·6 to 11·8 % H. From this fraction a very small amount of white crystals was separated melting at 49–50°, which appeared to be identical with those obtained in another series of experiments (see p. 1260), and which gave figures on analysis approximating to those required by ketostearic acid. The fraction obtained from one experiment (C, 74·03; H, 11·32 %) was converted into the zinc salt and this fractionally crystallised from boiling alcohol. The acid obtained from the least soluble portion gave figures on analysis agreeing with those for oleic acid: the composition of that from the most soluble part of the zinc salt agreed with that of a ketostearic acid (C, 72·55, 72·53; H, 10·61, 10·80 %) but, since it had an iodine value of 41, it still contained a considerable proportion of oleic acid, and we were unable to obtain from it either a satisfactory oxime or dinitrophenylhydrazone, nor did crystals separate on standing.

An attempt to calculate the composition of the oxidation product was made as follows. The acid derived from the soluble zinc salt contained 72·54 % C and 10·7 % H, and had an iodine value of 41. If the latter were due to oleic acid (C, 76·6; H, 12·05 %; I.V. 90), the following equations would give the percentage of carbon and hydrogen in the oxidation product.

$$41 \times 76{\cdot}6 + 49\ \mathrm{C} = 90 \times 72{\cdot}54, \text{ whence } \mathrm{C} = 69{\cdot}1\ \%$$
$$41 \times 12{\cdot}05 + 49\ \mathrm{H} = 90 \times 10{\cdot}7, \text{ whence } \mathrm{H} = 9{\cdot}6\ \%.$$

These figures agree most nearly with those required for the diketostearic

acid (C, 69·23; H, 10·26 %), so that although we have not yet succeeded in isolating this substance, it is possible that it is present.

Table I. *Results of oxidation of 4·05 g. oleic acid as Na salt by 33 cc. (20 vol.) H_2O_2 in absence of a catalyst.*

Wt. CO_2 g.	Volatile acids g.	Volatile neutral. Rothera's acetone test	Fraction water-ether-soluble g.	Fraction water-soluble ether-insol. g.	Fraction water-insol. ether-sol. g.	
					Acid	*Neutral*
0·10	Formic 0·09 Acetic 0·48	Positive	(*a*) 0·11 water-sol. acid (*b*) 0·05 alcohol-sol. acid	0·07	*Petroleum-soluble:* (*a*) oleic acid 1·71 (*b*) diketostearic acid ? 0·81 (*c*) α-ketodecoic or α-keto-nonoic 0·28 *Petroleum-insoluble:* Dihydroxystearic 0·46	0·3

In his work on the oxidation of the saturated fatty acids, Dakin found that the amount of oxidation was very little affected by increasing the proportion of hydrogen peroxide. In the series of experiments about to be described in which a cupric salt was used as catalyst, a large excess of peroxide was taken and an experiment was therefore carried out in which the same excess of peroxide was used but without the addition of the catalyst. Sufficient peroxide was now present to oxidise completely the whole of the oleic acid to carbonic acid.

Carbonic, formic and acetic acids now accounted for about 18 % of the carbon of the original oleic acid: the chief product was still a colourless oil, but the greater proportion of it was now insoluble in light petroleum. The proportion of dihydroxystearic acid was much greater and more of the oleic acid had undergone oxidation.

To sum up, whereas in Dakin's investigations on the higher saturated fatty acids about 10 % of the acid was attacked, the chief products being carbonic acid and the methyl alkyl ketone formed as decomposition products of the corresponding β-hydroxy-acid, when oleic acid is similarly treated about 50 % of the acid undergoes oxidation, a considerable proportion of it being converted to a fraction corresponding in composition with diketostearic acid; α-ketodecoic acid is formed and small amounts of dihydroxystearic acid. Carbonic, formic and acetic acids account for less than 10 % of the total carbon of the original oleic acid. Less than 5 % of the oxidation product consists of a neutral fraction in which a lactone of ketodihydroxystearic acid and an oil having the empirical formula required for a diketoheptadecane were present. The latter substance was not, however, definitely identified.

The oxidation of oleic acid with hydrogen peroxide in presence of a cupric salt.

When the oxidation of oleic acid is carried out in the presence of a cupric salt, the results differ widely from those just described. Oxidation proceeds

much more rapidly and at 95°, the temperature at which Dakin carried out his experiments, the carbonic acid formed accounts for the complete oxidation of more than 50 % of the total oleic acid, another 20 % being converted to acetic and formic acids. 70 % of the original acid has, therefore, been broken down to substances containing one or two carbon atoms. The fraction of residual oil which alone contains any product of a molecular weight approaching that of oleic acid is very small. In two experiments, in each of which 4 g. of oleic acid were taken, the residual oils insoluble in water were respectively 0·03 and 0·13 g. as compared with 3·7 g. in the corresponding experiment without the catalyst. Even at 60° the amounts of carbonic, formic and acetic acids correspond to a complete conversion of 20 % of oleic acid into these substances. At this temperature, the fraction derived from the aqueous reaction mixture by continuous extraction with ether contains the largest portion of the product, *i.e.* about 2·5 g. from 4 g. oleic acid, equivalent to the conversion of 40 % of the oleic acid. This fraction consists mainly of two substances:

(*a*) Crystals melting at 181–182° (0·5 g.) after being once recrystallised from water.

Analysis: C, 41·53; H, 5·23 %. Mol. wt. (by Ag salt), 132.

Calculated for $C_4H_6O_4$: C, 40·68; H, 5·08 %. Mol. wt. 118.

This substance was therefore identified as succinic acid.

(*b*) An oil (1·7 g.) to which the formula $C_8H_{14}O_4$ was assigned. Microanalysis of specimens obtained in two different experiments gave the following results:

C, 53·66, 53·41; H, 7·83, 7·97 %.

Mol. wt. (*a*) by titration, 166; (*b*) by iodoform estimation, 174, 189.

It was difficult to free this substance from all traces of succinic acid and the latter probably accounts for the low carbon percentage. The same substance was, however, subsequently obtained in a purer condition from the oxidation of caprylic acid (see p. 1259). A mixture of 90 % of the above acid with 10 % succinic acid would give 53·72 % C, 7·75 % H. Mol. wt. 162.

The formula $C_8H_{14}O_4$ was therefore adopted and the acid regarded as a 4-keto-7-hydroxycaprylic acid for the following reasons.

(1) It gave an iodoform reaction in the cold. This would be produced by the presence of a —CO— or —CH(OH)— group next to the terminal methyl group and its formation shows that oxidation must have occurred at the seventh carbon atom.

(2) The estimation of the $CH_3.CO$— group by its conversion to iodoform corresponded with a molecular weight of 174 and 189.

(3) The amount of substance extracted by ether from the reaction mixture when the experiment had been carried out at 95° was small but consisted chiefly of succinic acid. The product when the oxidation was carried out at 60° consisted mainly of the ketohydroxycaprylic acid, the proportion of succinic acid present being small. Some of the caprylic acid derivative

appeared, therefore, to have been converted into succinic acid at the higher temperature.

$$CH_3.CH(OH).CH_2.CH_2.CO.CH_2.CH_2.COOH \rightarrow COOH.CH_2.CH_2.COOH.$$

(4) In the oxidation of caprylic acid itself at 60° under similar conditions the same ketohydroxycaprylic acid and succinic acid were formed, so that in this case succinic acid must have come from the eight-carbon atom chain.

(5) The acid was not readily converted to a lactone and the hydroxyl group is not therefore in the γ- or δ-position.

(6) The presence of the —CO— or —CH(OH)-group in the α- or β-position could be excluded.

The amount of the ketohydroxycaprylic acid isolated when the oxidation was carried out at 60° corresponded to the conversion of almost exactly 70 % of the 8 terminal atoms of the oleic chain and it is improbable that the separation of this oxidation product was quantitative: it seemed most probable, therefore, that caprylic acid was formed from the oleic acid and subsequently underwent oxidation. The oxidation of caprylic acid was carried out under similar conditions and from the corresponding fraction of the product the same substance $C_8H_{14}O_4$ was isolated, together with succinic acid.

Its properties agreed with those described above.

Micro-analysis: C, 54·83; H, 7·57 %.

Calculated for $C_8H_{14}O_4$: C, 55·17; H, 8·05 %.

Mol. wt.: from titration 172; from an iodoform estimation 172. Theory 174.

Table II. *Showing results of the oxidation of 5 g. caprylic acid at 60° for 4 hours. Materials used; 32 cc. N NaOH, 110 cc. water, 15 cc. solution of $CuSO_4.5H_2O$ (2·73 %) and 350 cc. H_2O_2 (20 vols.).*

Product	Exp. I g.	Exp. II g.	Approximate % of total carbon calc. on mean of I–II	
Carbon dioxide:				
(*a*) during oxidation	0·50	0·33	—	
(*b*) during distillation	0·28	0·35	6·1	
Volatile acids:				
Caprylic (recovered)	0·37	0·55	9·3	Mol. wt. by titration {147 155
Acetic, formic	1·03	0·69	10·4	Mol. wt. by titration {79 49
Soluble in water and in ether:	3·99	3·25	59·8	Calc. as containing 55 % C
Succinic	1·80	—	—	Small amount crystals
Ketohydroxycaprylic				M.P. 178–180°. For analysis of ketohydroxycaprylic acid see above
Sparingly sol. in water, sol. in alcohol	1·03	—	—	Contains a lactone
Soluble in water, insoluble in ether	0·40	0·18	2·4	Mol. wt. (dibasic) 82. Glycollic and glyoxylic absent. Chiefly oxalic
Insoluble in water, soluble in ether:				
(*a*) residual oil	0·10	0·70	6·5	Mol. wt. by titration {178 171
(*b*) extracted by ether from charcoal	0·14	0·14	—	Titrates as lactone
		Total	94·5 of total carbon	

When the oxidation of the oleic acid was carried out at 60° about 20 % of it was converted to a mixture of oxidised derivatives of stearic acid, varying somewhat in different experiments. These were separated into products respectively insoluble and soluble in light petroleum, and the following fractions were separated and analysed. The residual oil varied in composition in different experiments and we did not obtain sufficient completely to purify the various fractions, or to identify them satisfactorily.

Table III. *Composition of fractions of water-insoluble oil obtained by oxidation of oleic acid at 60° in presence of a cupric salt, determined by micro-analyses.*

(1) Crystals M.P. 48–50°, soluble in light petroleum
C, 73·57; H, 11·73 %. Mol. wt. by titration 302.
Calculated for ketostearic acid: C, 72·50; H, 11·41 %. Mol. wt. 298.
M.P.: 9-ketoacid 83°; 10-ketoacid 74–76°

(2) Crystals M.P. 84–86°, insoluble in light petroleum
C, 67·44; H, 11·25 %.
Calculated for dihydroxystearic acid: C, 68·34; H, 11·39 %.
M.P.: low-melting form 98–99°; high-melting form 130°

(3) Oil soluble in light petroleum
C, 67·59; H, 10·41 %

(4) Oil insoluble in light petroleum
C, 66·58; H, 9·98 %. Mol. wt.: acid from Ag salt 348; by titration 358. Oil titrates like a lactone.
Calculated for lactone of ketotrihydroxystearic acid: C, 65·87; H, 9·79 %. Mol. wt. acid 346

(5) Oil
C, 61·48; H, 9·98 %

Method of experiment.

4 g. of pure oleic acid were neutralised with 95 % of the theoretical amount of *N* NaOH, and dissolved in 210 cc. of water to which 24 cc. of a 2·73 % solution of $CuSO_4$, $5H_2O$ had been added. The solution was contained in a three-litre flask fitted with a reflux condenser, to the top end of which was connected (*a*) an inlet tube provided with a clip through which air or nitrogen could be passed into the liquid in the flask, and (*b*) an exit tube leading to a series of flasks containing known volumes of standard baryta solution. This was then heated on a water-bath at the required temperature. 560 cc. of 20 volume hydrogen peroxide, previously made neutral, were added, and the flask well shaken. The inlet tube was closed and the CO_2 evolved collected in the baryta flasks. After 3 to 4 hours the heating was stopped, 5 cc. of concentrated HCl or H_2SO_4 were added to the reaction mixture and a current of nitrogen passed through to drive off CO_2, which was absorbed by the standard baryta.

The reaction mixture was filtered through a wet filter-paper which was subsequently dried and extracted with ether, any oil insoluble in water being thus separated. The filtrate was made alkaline to litmus, charcoal added and left to stand overnight so that any excess of hydrogen peroxide was destroyed. The charcoal was removed by filtration and when dry extracted with ether to remove any product which it might have absorbed. The filtrate was acidified

Table IV. *Showing results of oxidation of oleic acid by H_2O_2 in presence of a cupric salt.*

Table IV. *Showing results of oxidation of oleic acid by H_2O_2 in presence of a cupric salt.*

	CO_2		Volatile acids		Volatile neutral. Na nitroprusside test for acetone	Extract sol. in water and in ether g.	% of total C	Extract sol. in water, insol. in ether g.	% of total C	Oil insol. in water, sol. in ether g.	% of total C
At 60°	Wt. (g.)	% of total C	g.	% of total C							
Exp. I. 3½ hrs.											
4 g. oleic acid as Na salt, 560 cc. H_2O_2 (20 vol.)	During oxid. 0·67 ,, dist. 0·30 0·97	8·6	Formic 0·10 Acetic 0·68	9·7	Positive	Total 1·56 *Easily sol. in water* 0·92 (*a*) 4-keto-7-hydroxy-caprylic acid (*b*) succinic acid *Sol. in alcohol* 0·38	—	Total 0·49 (contains oxalic and glycollic)	4·6 approx.	Total 0·86	18·9 approx.
Exp. II. 3½ hrs.											
4 g. oleic acid as Na salt, 560 cc. H_2O_2	During oxid. 1·04 ,, dist. 0·37 1·41	12·6	Formic 0·09 Acetic 0·65	9·4	Positive	Total 2·46 *Easily sol. in water* 2·15 (*a*) ketohydroxycaprylic acid 1·69 (*b*) succinic acid 0·46 *Sol. in alcohol* 0·10	36·2	Total 0·76	7·2 approx.	Total 0·83	18·2
Exp. III. 3½ hrs.											
4·86 g. oleic acid as NH_4 salt, 560 cc. H_2O_2	Not estimated	—	Formic 0·25 Acetic 1·11	13·7	Positive	Total 2·79 *Easily sol. in water* 2·59	—	Total 0·83	6·5 approx.	Total 0·63	11·4
Exp. IV. 5 hrs.											
4·26 g. oleic acid as NH_4 salt, 560 cc. H_2O_2	During oxid. 0·65 ,, dist. 0·22 0·87	7·2	Formic 0·08 Acetic 0·79	9·7	Positive	Total 2·93	—	—	—	Total 0·53	10·9
At 95°											
Exp. V. 3½ hrs.											
4·1 g. oleic acid as Na salt, 560 cc. H_2O_2	During oxid. 5·75 ,, dist. 0·45 6·20	53·9	Incompletely acidified, not estimated	—	Positive faint	Total 0·40 *Easily sol. in water* 0·25 *Sol. in alcohol* 0·15	—	Total 0·27	2·5 approx.	Total 0·13	2·8
Exp. VI. 3½ hrs.											
4·03 g. oleic acid as Na salt, 560 cc. H_2O_2	During oxid. 5·03 ,, dist. 0·55 5·58	50·5	Formic 0·01 Acetic 1·45	18·6	Positive	Total 0·76 *Easily sol. in water* 0·55 (*a*) ketohydroxy-caprylic acid 0·07 (*b*) succinic acid 0·48 *Sol. in alcohol* 0·09	7·6	Total 0·33	3·1 approx.	Total 0·03	0·7

and steam-distilled, the apparatus being arranged so that any CO_2 given off could be absorbed and estimated.

The distillate was neutralised and distilled to remove any neutral volatile products, the neutral distillate being tested for acetone and aldehyde. The remaining solution was evaporated to dryness and the sodium salts of the volatile acids obtained. The percentage of formate in this was estimated by the mercury method. After steam distillation, the residual non-volatile liquor was evaporated to a small bulk and subjected to continuous ether extraction for several days. The total extract obtained was evaporated to dryness and dried in a vacuum desiccator until of constant weight. The golden-brown oil remaining was extracted first with water and then with 96% alcohol, and the residues from these two extracts were separately examined.

The aqueous solution, after it had been thoroughly extracted with ether, was treated with H_2S to remove traces of copper and then evaporated to dryness and extracted with absolute alcohol. The residue contained in the alcohol was weighed and examined for glycollic, glyoxylic and oxalic acids.

Discussion.

When oleic acid is treated with hydrogen peroxide in the presence of a copper catalyst, it appears that oxidation first takes place at the 9th and 10th carbon atoms and then proceeds further in the 18 carbon chain, the position of the carbon atoms next attacked not having been definitely determined; since a lactone was obtained it seems probable that oxidation had taken place in the γ- or δ-position.

The oxidised chain then breaks up, but neither azelaic nor suberic acid could be detected. Succinic and oxalic acids were the only dibasic acids isolated, only a trace of the latter being present.

The eight terminal carbon atoms of the oleic chain are broken off and the remaining part of the chain appears to be completely broken up, succinic, carbonic, formic, acetic, glycollic and oxalic acids having been identified. The amount of succinic acid formed during the oxidation must be considerably greater than that actually isolated, for it has been shown that in an acid reaction mixture succinic acid is rapidly oxidised under the conditions of the experiment [Battie and Smedley-MacLean, 1929]. During the oxidation of the oleic acid in alkaline solution probably comparatively little of the succinic acid is attacked, but after the acidification of the reaction mixture, excess of the peroxide still being present, considerable oxidation of the succinic acid will occur while the carbonic acid is being removed from the solution.

The eight terminal atoms of the oleic chain undergo oxidation, behaving apparently as caprylic acid itself behaves under similar conditions, and the 4-keto-7-hydroxycaprylic acid first formed is broken down at a higher temperature, there being evidence that succinic acid is formed as a product of this decomposition.

In the absence of the copper catalyst it is possible that a very small amount

of a ketone may have been formed such as would be expected if the ketostearic acid first formed had undergone β-oxidation, but the evidence for it was not definite, and in any case the amount was very small. The direct evidence for the β-oxidation of the higher fatty acids rests exclusively on experiments on the oxidation of stearic, palmitic and myristic acids by means of hydrogen peroxide at a temperature very little below the boiling-point of water. Experiments furnishing evidence that the animal body can directly bring about β-oxidation have all been carried out with acids containing ten or less carbon atoms. The experiments of Embden and Marx [1908], in which the liver was perfused with solutions of salts of the lower fatty acids containing up to ten carbon atoms, showed that one molecular proportion of the even-numbered fatty acids produced from 10 to 17 % of a molecule of acetone. The highest proportion (17 %) was produced from a molecular proportion of butyric and decoic acids respectively, which is in accordance with the view that one molecule of acetoacetic acid comes from one molecule of fatty acid, being derived probably from the four terminal carbon atoms. Actually, small amounts of acetone were also derived from the odd-numbered acids, a molecular proportion of nonoic acid furnishing 4 % of a molecule of acetone.

Again, the classical feeding experiments of Knoop were carried out with acids containing not more than six carbon atoms.

Leathes has urged that the first stage in the utilisation of the fatty acids consists of their desaturation in the liver. The results of the experiments *in vitro* now described show that when oleic acid is oxidised under these comparatively mild conditions, an eight-carbon chain fatty acid remains intact and undergoes further oxidation, but that the chain from the carboxyl group to the double bond is completely broken down into substances containing not more than two carbon atoms with the possible exception of succinic acid. The actual isolation from oleic acid of caprylic acid derivatives disposes of the objection raised by Leathes and Raper [1925, p. 194] that oleic acid would first be broken down into pelargonic and azelaic acids, and that as these contain odd numbers of carbon atoms they would not be expected to furnish acetoacetic acid.

The evidence now presented from laboratory experiments has been obtained under conditions probably more nearly approaching those existing in the body than those used in previous work on the oxidation of oleic acid. The action of a mild oxidising agent such as hydrogen peroxide at 60° is not very far removed from conditions *in vivo* and even copper is known to occur in marked amount in liver tissue [Morrison and Nash, 1930].

The fact that the introduction of a cupric salt as catalyst makes such a marked difference in the course of the oxidation of fatty acids by hydrogen peroxide, in the case of caprylic acid replacing the β-oxidation described by Dakin by γ-oxidation and in the case of caprylic and oleic acids producing a considerable proportion of succinic acid, is of some interest when its biological application is considered.

The wide distribution in the body of the powerful specific enzyme succinoxidase [Batelli and Stern, 1911; Thunberg, 1909] has so far found no sufficient explanation, for, as Clutterbuck [1927] has pointed out, its only function appears to be to deal with the comparatively small amount of succinic acid derived from glutamic acid.

The conversion of 50 % of butyric into succinic acid [Cahen and Hurtley, 1917], the production of γ- and δ-keto-acids from the higher saturated fatty acids [Clutterbuck and Raper, 1925] and the conversion of the δ-keto-acids to succinic acid led Clutterbuck to suggest succinic acid as a possible intermediary between fat and carbohydrate.

The experiments now described show that under mild conditions of oxidation succinic acid may be formed in considerable quantity from oleic acid, and in the body this product would be further oxidised by the succinoxidase of the liver and muscles to malic acid.

A considerable body of evidence is accumulating that there is a widespread mechanism for dealing with the oxidation of succinic acid, not only in the animal body but also in lower organisms. Stent, Subramaniam and Walker [1929], for instance, have shown that *Aspergillus niger* contains enzymes by which succinic is converted through malic to oxaloacetic acid and point out that the conversion of the latter to pyruvic and lactic acids would form a bridge over which one might pass from fat to carbohydrate. They also found that when *A. niger* acts on butyric acid small amounts of succinic acid are formed; they appear to prefer the view that these are formed rather by dehydrogenation of acetic acid derived from the oxidation of the butyric acid than by direct oxidation of the butyric acid. We carried through an oxidation of acetic acid under conditions similar to those we used for the oxidation of oleic acid but were unable to extract any succinic acid from the product: therefore the succinic acid produced in our experiments is certainly not derived from acetic acid.

In all our experiments on the oxidation of oleic acid traces of acetone have been identified in the neutral volatile product by means of the sodium nitroprusside reaction, and in one case this has been definitely confirmed by the preparation of a crystalline derivative. It is possible that the source of the acetone might be the 4-keto-7-hydroxycaprylic acid, which might suffer the following degradation:

$$CH_3.CH(OH).CH_2.CH_2.CO.CH_2.CH_2.COOH \rightarrow CH_3.CO.CH_2.COOH \rightarrow CH_3.CO.CH_3;$$

or the caprylic acid residue might undergo oxidation, leaving a butyric acid residue which might then be oxidised further, or the acetone might be derived from the remaining part of the oleic molecule. In any case the amount formed is only just detectable and possibly represents the product of some alternative path of oxidation.

The evidence that β-oxidation of the lower fatty acids can take place in

the body is convincing, but it does not necessarily follow that this is the only method of oxidation of the saturated fatty acids nor that it is the method by which the unsaturated acids undergo oxidation. The classical feeding experiments of Knoop which have led to the general acceptance of the β-oxidation theory have the disadvantage that they had to be carried out on the phenyl derivatives of the acids. In this case it would be impossible for the terminal butyric residue to be converted to succinic acid, since it would be a benzyl and not a methyl group that would be available for oxidation. Obviously, considerable caution must be exercised in accepting conclusions based on acids in which the terminal methyl is replaced by a benzyl group.

It is possible that alternative paths of oxidation exist in the body, so that the acids may be broken down either by a process of β-oxidation leading to the production of acetoacetic acid, or by a process involving γ-oxidation such as is now shown to occur in the laboratory with caprylic acid leading to the formation of succinic acid.

It may be that a catalyst exists in the body playing the part of the cupric salt *in vitro*, and that it is the failure of this catalyst to function effectively in starvation and in diabetes which leads to the production of large quantities of the acetone bodies.

Perhaps the solution of that mysterious problem, the manner in which "fat burns in the fire of carbohydrate" is to be sought in the effect of a liver stored with glycogen upon the oxidative mechanism. It is not impossible that the determining factor as to whether β- or γ-oxidation may occur is to be looked for in the nature of the liver cells and that the particular condition of the cell may be connected with its glycogen content.

In any case the possible degradation of oleic acid through succinic rather than through β-hydroxybutyric acid finds some support from the results now brought forward.

We are now investigating the influence of the copper catalyst on the oxidation of the higher saturated fatty acids by means of hydrogen peroxide.

Summary.

1. When the oxidation of oleic acid was carried out by hydrogen peroxide at 95° under the conditions used by Dakin in his experiments on the oxidation of the higher saturated fatty acids, a fraction, agreeing in composition with that of diketostearic acid, was formed, and evidence of the presence of an α-keto-derivative of nonoic or decoic acid was obtained. Some dihydroxystearic acid was also present.

2. The carbonic, acetic and formic acids produced corresponded to the complete oxidation of from 5 to 10 % of the total oleic acid. Increasing the proportion of hydrogen peroxide increased the proportion of dihydroxystearic acid present.

3. The addition of a small amount of cupric salt greatly increased the extent of the oxidation. At 95° with a large excess of hydrogen peroxide, more

than 70 % of the original oleic acid was completely broken down to carbonic, formic and acetic acids. Succinic and 4-keto-7-hydroxycaprylic acids were also identified.

4. When the oxidation in the presence of a cupric salt was carried out at 60°, the amounts of carbonic, formic and acetic acids produced corresponded to the complete oxidation of about 20 % of the total oleic acid. The product obtained in greatest amount was a derivative of caprylic acid to which the formula 4-keto-7-hydroxycaprylic acid has been assigned. A considerable proportion of succinic acid was also present. A small quantity of an oil, insoluble in water, consisted of a mixture of various oxygenated derivatives of stearic acid, the composition of which varied in different experiments.

5. Among the products of oxidation of caprylic acid were succinic acid and the same 4-keto-7-hydroxycaprylic acid as was previously obtained from oleic acid.

6. The bearing of these results on the theory of β-oxidation is discussed.

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CXLI. SOME BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF COPPER IN ANIMAL NUTRITION.

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WITHIN recent years our conception of the mineral nutrition of animals has had to be broadened to take account of certain "trace" elements which have been found to be widely distributed in the vegetable and animal kingdoms. These elements, *e.g.* manganese, iodine, zinc, copper, *etc.*, occur only in minute traces but their possible physiological significance cannot be neglected.

The claim that copper is essential for the utilisation of iron in haemoglobin building [Waddell *et al.*, 1928] arouses particular interest in this element. The work outlined below was undertaken with a view to obtaining further information on this and cognate subjects.

I. THE OCCURRENCE OF COPPER IN NATURE.

(*a*) *The vegetable kingdom.*

The occurrence of copper in vegetable materials has been investigated mainly with the object of discovering the amounts of this element in materials used as foodstuffs. Such investigations date as far back as 1816 [Meissner, 1816] but the greater number have been carried out within the last eleven years [Maquenne and Demoussy, 1920; Fleurent and Levi, 1920; Guérithault, 1920; Quartaroli, 1928; Elvehjem and Hart, 1929; Lindow, Elvehjem and Peterson, 1929, *etc.*]. The data available show that copper occurs very widely in foodstuffs, the amounts varying somewhere between 4 and 20 mg. per kg. dry matter, but the copper content may be affected by soil conditions [Webster and Jansma, 1929] and increased by manurial treatment of the crop [Elvehjem and Hart, 1929].

Maquenne and Demoussy [1920] have shown that, while copper occurs throughout all parts of the same plant, the concentration is greatest in buds, young leaves and cell sap. This has been confirmed to some extent by McHargue [1926], whilst Javillier and Guérithault [1927] found that the concentration of copper in the germs of seeds was much greater than in the endocarp.

Apart from these observations on the occurrence of copper in foodstuffs and on the concentration in young parts of plants, no evidence has been found of a definite function of copper in plant life.

In view of the connection, in animal life, between copper and the porphyrin pigment haemoglobin, it was decided to determine whether the copper content of different parts of plants bore any relation to the chlorophyll content. Comparison was therefore made of the copper contents of the root, the stem and the leaf of plants such as turnips and between the outer and the inner leaves of cabbages and lettuces. Results are shown in Table I. The second column refers to a second series of analyses.

The analytical method for copper used throughout the work reported in this paper was that of Biazzo [1926] as modified by Elvehjem and Hart [1929]. The only further modification employed was the addition of sodium phosphate to the solutions of the ashed materials in order to avoid interference by traces of iron.

Table I. *Comparison of the copper contents of aerial and root portions of plants.*

Plant	Part analysed	Mg. Cu/kg. dry matter	
Leek	Leaves	9·8	—
	White edible portion	5·8	—
Lettuce	Outer leaves	18·7	15·8
	Inner leaves	25·8	25·4
	Centre stalk	12·6	—
Cabbage	Outer green leaves	4·7	4·8
	Inner white leaves	4·0	5·8
	Centre vein of inner leaves	2·1	2·9
	Centre vein of outer leaves	3·1	
	Centre stalk of plant	3·4	—
Carrot	Leafy part (excluding lower leaves)	10·7	10·1
	Stalks of leaves	—	5·0
	Red part of root	4·5	5·4
	Yellow part of root	—	5·5
Beetroot	Leaves	10·2	12·6
	Stalks	6·8	8·4
	Roots	6·8	6·6
Potato	Stalk	11·2	—
	Tuber	5·4	—
Turnip	Leaves	7·1	—
	Stalks	3·0	—
	Roots	4·4	—
Garden pea	Mature old seed	7·3	—
	Young green seed	8·6	—
	Pods of young seed	5·4	—
	Leaves	10·6	—

		Mg. Cu/kg. dry matter
Average (excluding cabbage and lettuce):	Leaves	9·9
	Stalks	6·7
	Roots	5·5

It is seen that there is more copper in the aerial parts of plants than in the roots. This is probably not connected with chlorophyll content as the white inner leaves of cabbages and lettuces contained as much (or more) copper as the outer chlorophyll-rich leaves. It is notable that in stems and pods, where a large amount of fibrous material is to be found, the copper content is lower.

(*b*) *The animal kingdom.*

The wide distribution of copper in vegetable foodstuffs suggests its occurrence in animal organisms, and data are available showing the presence of this element in insects, marine animals and land animals.

(i) *Insects and marine animals.* Information concerning the copper content of insects is not very definite. Muttkowski [1921] obtained a positive qualitative test in samples representing 34 different species and Haber [1926] showed qualitatively that copper occurs in the blood of *Blatella germanica*. Quantitative figures are available only for three species [Giunti, 1879; Aronssohn, 1911].

In the case of marine animals, on the other hand, a considerable number of copper determinations have been published by Rose and Bodansky [1920] and by Severy [1922]. It was shown that of 35 species the copper contents varied from about 60 mg./kg. dry matter in the oyster to traces in the whale, the amounts diminishing progressively in ascending from the comparatively simple to the more complex organisms.

The presence of copper in marine animals has been explained only in the case of those animals whose blood contains haemocyanin.

Analytical data obtained by the writer for the copper contents of insects and of marine animals are given in Table II.

Table II. *Copper contents of some insects and marine animals.*

			Mg. Cu/kg. dry matter	Mg. Cu in whole organ or tissue of one animal
Insect		Cockroach (*Blatta orientalis*)	32·4	—
		House fly (*Musca domestica*)	77·6	—
		Honey bee (*Apis mellifica*)		
		Whole worker	25·0	—
		Fully developed queen bee grubs	24·5	—
		Young queen bee grubs	22·7	—
		Royal jelly	17·8	—
Marine animals		Octopus*		
		Skin	108·3	—
		Muscle	10·9	—
		Ink sac	914	8·8
		Ink (small sample)	No copper detectable	
	Tunicate	Mantle	10·9	0·020
	Lobster*	Liver	25·1	0·20
		Eggs	83·2	—
		Gonad	21·0	—
	Herring	Liver	14·2	0·008
		Skin	2·3	0·004
		Flesh	2·3	0·039
		Female reproductive organs	2·1	0·024
		Male „ „	2·3	0·022

* Samples washed with many changes of saline and finally with pure distilled water to free from traces of haemocyanin.

The figures for insects call for small comment except that it is of interest that the copper percentage of the queen bee grub is higher than that of royal jelly, its sole source of food.

Referring to marine animals, it is seen that there is a very high proportion and amount of copper in the ink sac of the octopus. Such accumulation of copper in the ink sac is worthy of note particularly as the ink contains no appreciable quantity of this element. Reference will be made later to observations of a catalytic influence of copper on the oxidation of *l*-3:4-dihydroxyphenylalanine to melanin. Since the ink of such sea animals contains melanin it is possible that, in the ink sac, copper has a function in the formation of the ink.

(ii) *Copper in land animals.* The occurrence of copper in land animals has been reported, but only in the case of the rat is there reliable information regarding the distribution of copper throughout the various organs of the body [Lindow, Peterson and Steenbock, 1929]. Some figures are available for different organs of man, the ox and the horse, but the data are scanty and the practice of taking different organs from separate animals has not permitted of definite conclusions being drawn regarding the distribution of copper throughout the body. The livers only, of a number of other species, have also been investigated for copper content.

In the present work, copper was determined in the organs from 12 different species of animals and the results are reported in Table III. The figures in a

Table III. *Copper in organs of different species expressed as mg./kg. dry matter.*

Species	Description	Liver	Heart	Lungs	Spleen	Kidney	Pancreas	Brain	Flesh	Skin	Hair
Human	Adult*	24·9	—	—	5·2	17·5	4·3	17·5	—	—	—
Bovine	Adult†	77·0	15·6	5·3	2·9	19·7	3·8	—	—	—	—
Bovine	Calf newly born	470·0	14·8	4·9	4·8	15·7	5·5	—	4·8	—	—
Bovine	Foetus	262·8 (16·3)	10·4 (0·086)	3·6 (0·115)	5·4 (0·060)	8·5 (0·087)	— —	— —	2·9 —	2·1 (0·458)	— —
Sheep	Adult	236·6 (60·0)	17·9 (0·68)	9·6 (0·75)	5·0 (0·08)	17·8 (0·36)	7·7 (0·04)	— —	— —	— —	— —
Horse	Adult†	14·8 (30·5)	17·6 (11·9)	6·8 (10·0)	3·2 (1·7)	28·9 (8·2)	— —	— —	— —	— —	— —
Pig	Adult†	41·3 (19·9)	14·9 (0·86)	5·3 (0·55)	6·0 (0·13)	21·1 (1·35)	— —	— —	— —	— —	— —
Pig	Few days old	232·8 (2·0)	12·8 (0·020)	3·4 (0·010)	6·7 (0·0013)	14·7 (0·050)	— —	— —	— —	— —	— —
Dog	9 days old	98·2 (0·320)	17·4 (0·006)	6·2 (0·005)	— —	14·2 (0·009)	— —	8·5 (0·012)	— —	9·9 (0·076)	22·7 (0·056)
Cat	Adult†	25·3 (0·534)	14·4 (0·039)	3·8 (0·033)	5·2 (0·016)	10·1 (0·075)	— —	14·6 (0·079)	2·3 —	4·2 (0·433)	11·9 (0·790)
Guinea-pig	Adult	17·0 (0·102)	21·2 (0·005)	9·5 (0·006)	— —	19·9 (0·016)	— —	— —	— —	— —	— —
Rabbit	Adult	9·2 (0·17)	22·3 (0·023)	8·1 (0·018)	— —	13·7 (0·040)	— —	— —	— —	— —	— —
Rat	About 90 g. wt.†	10·0 (0·016)	27·8 (0·0023)	9·5 (0·0015)	8·1 (0·0016)	22·6 (0·005)	— —	10·2 (0·003)	3·8 —	7·3 (0·025)	14·8 (0·014)
Badger	Adult†	21·7 (2·09)	12·8 (0·112)	5·6 (0·100)	3·0 (0·019)	9·4 (0·094)	— —	10·8 (0·089)	— —	3·2 —	24·7 —
Domestic fowl	Adult†	12·4 (0·081)	14·9 (0·014)	2·4 (0·003)	— —	11·7 (0·018)	— —	— —	— —	Feathers —	4·9 —
Average:			16·8	6·0	5·0	16·4	—	—	—	—	—
Range of variation:			10·4–27·8	2·4–9·5	2·9–8·1	8·5–28·9	—	—	—	—	—

* Average of 3 series of analyses. † Average of 2 series of analyses.

horizontal line refer to the organs from one and the same animal or group of animals and the total amount of copper, expressed in mg. in the entire organ from one animal, is given in brackets. These figures may be used in discussion of the distribution of copper throughout the various organs of animals from any one species without encountering any effect due to individuality or diet.

The averages and ranges of variation of the copper content of heart, lungs, spleen and kidneys for all species are also given in Table III.

Although the ranges of variation for heart, lungs, spleen and kidneys seem rather wide it should be noted that the extreme figures occur in only a few cases, most of the figures falling within 50 % of the average. The differences in copper content between these different organs therefore appear to be real differences and not due to chance variation.

Further investigation was undertaken to find the variation of copper in any one organ due to individuality, under controlled conditions of feeding. In this case, copper was determined in the livers of normal newly born piglings, whose mothers had all been receiving the same diet. The averages and range of variation for 15 livers are given in Table IV and figures for iron content are also included.

Table IV. *Copper and iron content of infant pig livers.*

	Mg. per kg. dry matter		Mg. in whole liver	
	Copper	Iron	Copper	Iron
Average	241·4	1390	1·15	6·14
Range	50·8–465·7	580–1910	0·11–2·00	2·63–12·12

Again, although the ranges of variation are rather large, only in 4 of the 15 cases did the total copper in the liver vary more than 50 % from the average. The proportion of copper in the dry matter varied in a similar manner. There was no correlation between the iron and copper contents, but the variations in respect of iron were as great as those in respect of copper.

In all the organs so far dealt with, then, the variation of copper content was too great to assign a definite value for it in a given organ. At the same time the differences between the different organs were significant, and the distribution of copper throughout the organs was substantially the same for all species examined. Classification of organs according to descending value for either proportion of copper or total weight of copper in the organ gave liver, kidneys, heart, lungs, pancreas, spleen.

Analyses of organs not referred to in this list or in Table III are given in Table V. It is seen that copper is present in all the parts of animals (and of plants) examined, and it is possible that this element may fill a small corner in the composition of protoplasm. Fox and Ramage [1931] have made a similar suggestion from spectrographic examination of animal tissues.

A general division of the parts of animals may be made, giving two classes

(*a*) those containing a high proportion of copper;

(*b*) those containing a low proportion of copper.

Table V.

Species	Sample	Mg. Cu/kg. dry matter
Human	Hair	Av. 2 15·1
Bovine	Stomach wall	Av. 3 6·5
	Parotid salivary gland	Av. 2 6·6
	Horn	3·9
	Hoof	2·1
	Fatty bone marrow	0·16
	Red bone marrow, calf	Av. 2 4·4
	Thymus, calf	3·5
Sheep	Thymus	6·5
	Lymph glands	10·8
	Digestive tract	9·0
Horse	Adrenal	18·0
Dog	Melanotic tumour	Av. 2 8·0
	Bile	71·0
Cat	Bile	Av. 2 22·7
Rat	Tongues	Av. 2 16·6
Domestic Fowl	Skins and scales of leg	4·7
	Proventriculus	12·8
	Gizzard	3·2
	Gizzard lining	Av. 2 27·0

In (*a*) are included liver, kidneys, heart, brain, hair, while (*b*) includes skin, lungs, pancreas, spleen and flesh.

It was thought possible that some function might be associated with the presence of copper in certain organs and tissues, and further investigations were made of the skin and hair and the liver.

Skin and hair. Appreciably high amounts of copper were found in the skin and hair of all animals examined. Some figures were given in Table III and further figures are given in Table VI.

Table VI. *Copper in skin and hair of animals. Mg. Cu/kg. dry matter.*

Species		Skin	Hair or Wool
Bovine	Epidermis	6·0	
	Dermis	2·3 (av. of 2)	
Sheep		2·8 (av. of 3)	8·3 (av. of 10)
Rabbit		6·6 (av. of 6)	15·4 (av. of 4)
Rat		9·7 (av. of 2)	12·2 (av. of 2)
Rats from same litters	At birth	18·6 (av. of 4) (0·0020)*	
	1 week old	14·8 (av. of 4) (0·0090)	
	2 weeks old	9·3 (av. of 4) (0·0105)	
	3 weeks old	13·7 (av. of 2) (0·036)	
Guinea-pigs from same litter	At birth	21·4 (0·070)	12·5 (0·029)
	1 week old	10·1 (0·035)	13·7 (0·031)
	2 weeks old	11·0 (0·036)	11·8 (0·034)

* Figures in brackets represent the weight in mg. of copper in the whole tissue or part from one animal.

The figures quoted indicate that, in rats, the amount of copper in the skin increased rapidly from birth and reached a fairly steady value at about three

weeks of age, while in guinea-pigs the amount of copper decreased from the value at birth and became constant at about one week old. Several other series of analyses were made of both these species in which the age limits were narrowed or lengthened, and the findings from these data, which for the sake of brevity are not cited here, were in complete accord with the typical results quoted in Table VI.

The presence of copper in rat skins in somewhat different amounts has previously been reported by Lindow, Peterson and Steenbock [1929]. These workers found that the average amount of copper in the skin and hair of an adult rat was 0·161 mg. and the proportionate amount was 3·57 mg. copper/kg. dry matter. According to them, feeding a high level of copper to rats for 7 weeks did not increase the copper content of the skins above this value.

The author has investigated the effect of the amount of copper in the diet on the copper content of rat skins with the following results.

Table VII. *Copper content of rat skins* (*average values*).

No. of rats	Copper in diet mg. daily	Time fed diet months	Skin		Hair		Skin and Hair	
			mg. Cu per skin	mg. Cu/kg. dry matter	mg. Cu per animal	mg. Cu/kg. dry matter	mg. Cu per animal	mg. Cu/kg. dry matter
4	0·011	9	0·054	6·9	0·022	9·5	0·076	8·1
3	0·050	9	0·048	6·9	0·021	13·8	0·069	8·2
3	0·075	7	—	—	—	—	0·064	4·4
1	7·5	1	—	—	—	—	0·078	—
3	7·5	7	—	—	—	—	0·113	6·8

A slight increase in the total copper content of the skin and hair occurs after prolonged feeding of high doses of copper, but the effect is not very marked. At the same time almost identical amounts of copper were found in the skins of rats fed such different levels of copper as 0·011 and 0·050 mg. daily for long periods. It is therefore unlikely that the skin is a path for excretion of copper.

The occurrence of somewhat uniform amounts of copper in the skin and hair of many species merited some investigation. No explanation can be offered in the case of the hair, though it is of interest to note (1) that in bovines the concentration of copper is greater in the epidermis than in the dermis (Table VI), and (2) that in the sheep, an animal which produces large amounts of wool, the copper content of the liver is much higher than in any other species. On the other hand, while Angora rabbits produce large amounts of hair, the copper content of the livers is of the same order as that of short-haired rabbits.

Some instances were noticed amongst rats and rabbits in which the copper content of the skins of black animals was higher than for white animals. While this was not always so, the relation of copper to pigmentary processes was considered and the effect of this element on the oxidation of "dopa" (*l*-3:4-dihydroxyphenylalanine) was tried. "Dopa" is a chemical precursor of melanin into which it is converted on oxidation. It has been used by Bloch [1927] and by Stewart and Percival [1930] as a histological stain for detecting "dopa"

oxidase in skin, and by Koller [1930] in studying the genetics of pigmentation in rabbits. The technique adopted was largely based on the work of Koller.

It was found that the oxidation of a solution of "dopa" by means of an extract of the skin of newly born rats, rabbits or guinea-pigs only proceeded slowly, maximum coloration at room temperature occurring after about 60 hours. The addition of copper to the solution materially accelerated the oxidation process, always in proportion to the amount of copper added, and the addition of 0·05 mg. of copper in 2 cc. of solution sufficed to give maximum coloration in 18 hours.

It is difficult to assess the significance of this catalytic action of copper, but it is of interest to consider the possibility already alluded to that the copper in the octopus ink sac may possibly be definitely related to the formation of the melanin pigment present in the ink. It is possible also, that in land animals the copper in the skin may play some similar part in the formation of melanin pigments.

A further connection between copper and pigmentation is to be found in the practices of bird fanciers for obtaining a deep yellow colour in canaries, in which copper sulphate, or other substances such as cayenne pepper, are fed to moulting birds. The new feathers are said to have uniformly deep yellow coloured edges and this was confirmed experimentally.

This effect of copper feeding is probably not due to an increased formation of a copper pigment such as turacin, the pigment of Turaco feathers [Church, 1892], since the copper content of canary feathers was found to be low compared with the content of turacin-bearing feathers. Analytical results are given in Table VIII.

Table VIII. *Copper content of feathers.*

	Mg. Cu/kg. dry matter
Canary (average of 3)	40
Turacus Hartlaubi medius	
Crimson, turacin-bearing, secondary wing feathers	2900
Black wing feathers (no turacin)	14

Thus, although there is a possibility that the copper in the skin may exercise some influence on pigmentary processes, evidence bearing on the subject is too scanty for any conclusions to be drawn.

Copper in liver. In every species of land animal studied the liver was found to contain a much greater amount of copper than any other organ. The ratio, weight of copper in liver to weight of copper in kidneys, was found to be, for the sheep 160, the horse 4, the cat 6, the rabbit 40, and of similar order for the other species. This observation also applies to such figures as have been published by other workers.

A notable feature is the high proportion of copper in sheep's liver; the average copper content of 9 livers was 323 mg. copper per kg. dry matter, the range being 212·5–497·5. Since there is in this country no well-established practice of dosing sheep with copper sulphate for internal parasites, this high

copper content cannot be due to such administration of copper. Figures of the same order have been reported by Aston [1911] for livers of normal New Zealand sheep.

The high proportion of copper in livers generally might be due to filtration, from the blood, of copper absorbed in the digestion of foodstuffs. In this connection, figures published by Lindow, Peterson and Steenbock [1929] showed that feeding rats 5 mg. copper (as $CuSO_4, 5H_2O$) per rat daily for 7 weeks increased the copper content of the livers of the rats nearly 20 times. The author has fed different amounts of copper to rats with the results given in Table IX.

Table IX. *Copper content of rat livers. (Average values.)*

Diet	Copper in diet mg. daily	Time fed diet months	No. of rats	Mg. Cu in whole liver	Mg. Cu/kg. dry matter
Stock	about 0·050	Extended period	21	0·020	12·8
Cow's milk only	—	,,	10	0·010	14·1
Synthetic	0·011	9	1	0·015	10·0
,,	0·050	9	3	0·023	13·9
,,	7·5	4	1	0·036	41·7
,,	7·5	5	8	0·049	19·6

The copper content of rat livers, then, depended on the amount of copper in the food but the storage observed when high proportions of copper were fed was markedly less than was observed by Lindow, Peterson and Steenbock [1929].

Storage of copper in livers of foetal animals. That a considerable storage of copper occurs in intra-uterine life has previously been shown in the case of the human [Morrison and Nash, 1930], bovine [McHargue, 1925, 1, 2] and porcine species [Powick and Hoagland, 1924; Hart *et al.*, 1930]. Similar observations have been made by the writer for a number of species and the results are collected in Table X.

Table X. *Copper in livers of adult, foetal and newly born animals. (Average values.)*

Figures in brackets give the number of analyses.

Species	Mg. Cu/kg. dry matter: Adult	Foetal	Newly born
Human	25 (3)	281 (1)	—
Bovine	70 (3)	171 (4)	550 (2)
Sheep	323 (9)	—	117 (3)
Pig	35 (7)	—	241 (15)
Guinea-pig	17 (3)	122 (3)	203 (4)
Rabbit	10 (7)	29 (3)	55 (4)
Rat	12·5 (23)	—	70 (3)
Dog	88 (3)	—	89 (2)

In the bovine, rabbit and guinea-pig species, where comparisons of the copper contents of livers of foetal and newly born animals were made, it was found that there was more copper in the liver of the newly born animal than in that of the foetal animal.

With the exception of sheep, and possibly dogs, the figures for the different species show a much higher percentage of copper in the liver of the newly born or foetal than in that of the adult animal. In all cases, even with lambs and puppies, it was found that there was a steady drop in the percentage of copper in the liver from birth onwards, throughout early infancy (Table XI).

Table XI. *Copper content of infant livers at different ages.*

Figures in brackets give the mg. Cu in the whole liver.

	Mg. Cu/kg. dry matter							
Species \ Age	At birth	3 days	1 week	2 weeks	3 weeks	4 weeks	8 weeks	12 weeks
Sheep	117·0	67·2	—	—	—	14·5	—	—
Dogs (same litter)	—	—	69·2 (0·209)	—	—	—	45·3 (0·534)	13·7 (0·287)
Rats (same litter)	87·8 (0·0045)	—	23·5 (0·0050)	14·5 (0·0063)	17·8 (0·022)	—	—	—
Guinea-pigs (same litter)	149·4 (0·171)		59·6 (0·102)	30·8 (0·042)	—	—	—	—

The observations recorded show that, of all organs the adult liver contains the highest proportion and absolute amount of copper, that the copper content of the diet influences the amount of copper in the adult liver, but not to a marked degree, and that the livers of newly born animals are provided with a large store of copper. This storage is possibly to provide the young animal with a supply of copper over the suckling period when only insignificant amounts of this element would be available in the diet of milk.

II. Copper and iron metabolism.

Copper and haemoglobin building.

As has been mentioned, one of the chief interests of copper arises from the claim by Hart *et al.* [1928] that, in the presence of this element inorganic iron may be used by rats for haemoglobin building.

Bunge [1902] stated that only "food" iron, *i.e.* iron in firm combination with organic compounds, as in haematogen, could be used by animals for haemoglobin building, and that the iron of iron salts or "inorganic" iron could not be used for this purpose. Even at the present day opinion is divided on this point and experimental evidence has been conflicting. However, the value of iron therapy in all types of human anaemias is well known and the success of this practice in curing Bush Sickness in New Zealand [Aston, 1921] and anaemia in young pigs in Scotland [McGowan, 1924] renewed interest in iron metabolism and particularly in haemoglobin production.

Waddell *et al.* [1928] have shown that newly weaned rats became anaemic in 4–6 weeks when their diets were restricted to cow's whole milk, and that the anaemia was not cured by supplementary feeding of purified "inorganic" iron. When copper was fed along with the "inorganic" iron, however, a rapid cure was effected. McHargue, Healy and Hill [1928], McCollum and Becker [1930],

Weichselbaum [1930] and Krauss [1930] have confirmed this effect of copper, but, on the other hand, Beard and Myers [1929] and Keil and Nelson [1931] found that purified "inorganic" iron alone would slowly cure the anaemia produced in rats by milk feeding. It has further been claimed by Titus and Hughes [1929] that manganese is as effective as copper in bringing about a cure of such an anaemia, and Beard and Myers [1929] also found that nickel, cobalt and germanium were as good supplements as copper. Mitchell and Miller [1929] obtained a maximal regeneration of haemoglobin when the ash of spinach was fed with a milk-iron diet and, since spinach ash contains many "trace" elements, these authors postulated that a complex of "trace" elements must be present in the diet along with iron for optimal utilisation of the last named element in haemoglobin building. Nevertheless, Weichselbaum [1930] obtained no haemoglobin regeneration when iron *plus* manganese or iron *plus* cobalt was fed to rats, and Krauss [1931] found no better effect with iron *plus* copper *plus* manganese than with iron *plus* copper. Again Waddell, Steenbock and Hart [1929] reported no effect with the twelve "trace" elements zinc, chromium, germanium, nickel, cobalt, lead, antimony, cadmium, tin, mercury, arsenic and manganese, fed singly or all together as supplements to a milk-iron diet.

All the work cited above has been concerned with the supply to, and utilisation of iron by, rats fed a basal diet of milk. Drabkin and Waggoner [1930] studied the effect of protein on haemoglobin regeneration. They found that rats made anaemic with milk diets were brought to normal with great rapidity when the diet was changed to a solid one containing iron but practically devoid of copper. Drabkin and Miller [1931] observed relief from anaemia, when arginine or glutamic acid was used as a supplement to the milk diet, while no success was recorded from feeding histidine. These authors believed that the anaemia produced by milk feeding was not due to a deficiency of iron but to deficiency of the pyrrole nuclei necessary in formation of the porphyrin moiety of haemoglobin.

From this brief survey it is seen that, while the effect of copper in promoting the utilisation of iron in haemoglobin regeneration has received considerable support, it has not been thoroughly established that copper is essential. A study has therefore been made of haemoglobin building from "inorganic" iron, alone or with various supplements.

Rats were used throughout the investigations and the method first employed, due to Waddell *et al.* [1928] was as follows. Anaemia was produced by feeding newly-weaned animals a diet consisting solely of cow's whole milk, and supplements were then fed individually to the rats and their effects measured by response in haemoglobin content of the blood.

Haemoglobin was determined with the Haldane haemoglobinometer, and on this scale the normal haemoglobin content of rat's blood is 80–90 %. Blood samples were obtained by puncturing a coccygeal artery or vein on the ventral aspect of the tail. The advantage of this procedure over the usual one of

snipping a small piece from the end of the tail is that the flow of blood can be regulated according to the condition of the animal by making a puncture near the base or near the tip of the tail.

The conditions of experimentation demanded rigid exclusion of all possible sources of metallic contamination, and special cages were therefore constructed from glass battery jars 13″ × 12″ × 9″ high. A false bottom of perforated celluloid allowed drainage of urine, whilst sufficient ventilation was provided by perforating the celluloid plate which closed the front of the cage. The milk used in feeding was drawn directly from the cow into an earthenware jar and the rats were fed from glass vessels.

It was found that deaths occurred very early when newly weaned rats were fed on the diet of cow's milk. In a number of experiments, 130 rats were used and of these 64 died after 2–5 weeks' milk feeding, the average time of survival being 3·2 weeks. After about 5 weeks' feeding the blood of the remaining animals contained low amounts of haemoglobin and these were given supplements. The iron used was purified by treatment in acid solution with H_2S, followed by several precipitations from acid solution by ammonia. For feeding purposes it was nearly neutralised with pure NaOH and for injection purposes the osmotic pressure was adjusted with pure NaCl. The response in haemoglobin content of the blood is shown in Fig. 1.

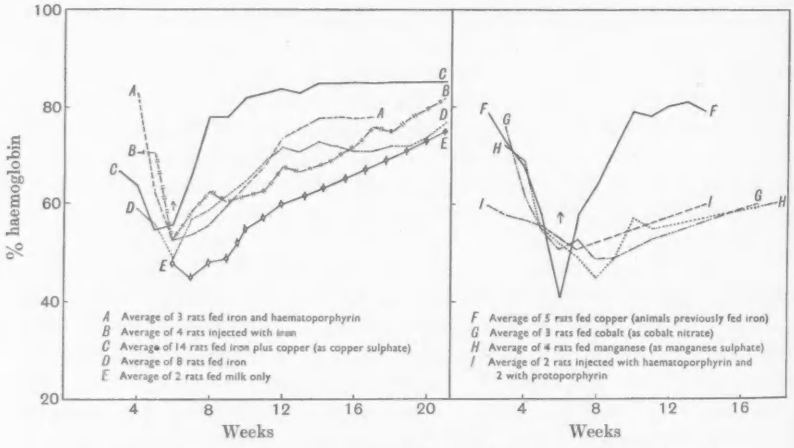
% haemoglobin
100
80
60
40
20
4
8
12
16
20
Weeks
A Average of 3 rats fed iron and haematoporphyrin
B Average of 4 rats injected with iron
C Average of 14 rats fed iron plus copper (as copper sulphate)
D Average of 8 rats fed iron
E Average of 2 rats fed milk only
F Average of 5 rats fed copper (animals previously fed iron)
G Average of 3 rats fed cobalt (as cobalt nitrate)
H Average of 4 rats fed manganese (as manganese sulphate)
I Average of 2 rats injected with haematoporphyrin and 2 with protoporphyrin

Fig. 1. Haemoglobin curves of rats fed milk diets from 0 weeks with various supplements from ↑.

The effect of copper feeding is at once obvious, for the rate of haemoglobin regeneration in rats fed this element is much more rapid than that observed when any other supplements were used. Curve *F* is of special interest. The animals to which it refers had previously been cured of anaemia by prolonged feeding of iron supplements and it was to be expected that considerable

quantities of iron would be stored in the liver. This was shown by comparing the iron contents of the livers of rats treated similarly up to the point marked ↑ on the curve, with those of rats fed milk alone. The following average figures were obtained.

	Mg. Fe in whole liver	% Fe in dry matter
Rats fed milk *plus* iron (long period)	2·493	0·158
Rats fed milk alone	0·917	0·078

The rats became anaemic when iron supplements were stopped notwithstanding this store of iron. When copper alone was fed, a rapid regeneration of haemoglobin followed, and it is therefore probable that the copper feeding caused the utilisation of the stored iron. After some time the animals were killed, and the iron content of the livers was determined. An average figure of 1·183 mg. Fe per liver or 0·127 % was found. The reduced quantity of iron supports the suggestion that copper feeding caused utilisation of the stored iron.

In view of these results it would appear that:

1. Newly weaned rats when fed a diet consisting solely of cow's whole milk developed a condition of anaemia very rapidly.

2. While a large proportion of the rats died if the diet was continued, slow recovery occurred in a small number of cases.

3. Additions of inorganic iron and copper to the diet prevented death and markedly hastened the recovery from anaemia.

4. Injection of iron, protoporphyrin or haematoporphyrin prevented death but had very little effect on the time of recovery.

5. Supplementary feeding of iron, manganese, cobalt or haematoporphyrin prevented death but had very little effect on the time of recovery.

These conclusions must, however, be considered in the light of certain complicating factors. Thus, the death rate was very high and, to investigate this further, 34 rats were fed a diet of cow's milk, and haemoglobin estimations made weekly from the commencement of feeding, for a period of 27 weeks. The results obtained may be arranged in three groups.

(*a*) Those rats which died soon after the milk diet was commenced and the haemoglobin of which was low. 8 deaths of this type occurred.

(*b*) Those rats which died after a longer period of milk feeding, and in which no marked lowering of haemoglobin occurred. 14 deaths of this type occurred.

(*c*) 12 rats which lived throughout the experiment exhibiting no dangerous lowering of haemoglobin.

In Fig. 2 are plotted haemoglobin curves: rat 628 typical of (*a*), rat 624 typical of (*b*) and the average of the 12 rats in (*c*).

The results indicate that there is some doubt as to whether a true nutritional anaemia was developed in rats fed on a diet of cow's milk. On the one one hand the long period of survival of the 12 rats in (*c*) and, on the other hand, the large percentage of early deaths both in (*a*) and in earlier experiments suggest the possibility that some type of epidemic caused these deaths rather

than that they were due to an uncomplicated condition of iron starvation. Pathological examination of some of the anaemic rats from the earlier experiments was kindly made by Dr J. P. McGowan. The blood contained low

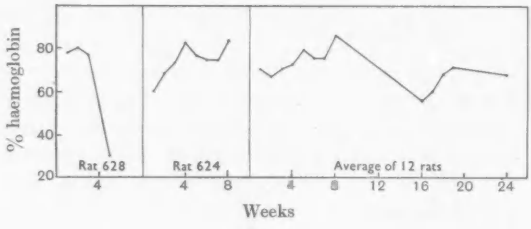

Fig. 2.

percentages of haemoglobin, but the bone marrow was hyperplastic, and marked reticulocytosis occurred in all cases. These conditions do not correspond with those of aplastic anaemias such as occur in iron deficiency and rather suggest an anaemia having as its characteristic feature a hyperplastic condition of the bone marrow. My attention was then drawn by Dr McGowan to the prevalence in rats of infections with Gärtner's bacillus and *Bartonella muris*.

Many of the animals which died in an acutely anaemic condition had *post mortem* symptoms corresponding to those in cases of death from *B. gärtner* [Boycott, 1911] and this organism was isolated[1] from the intestine of one of the animals.

Infection by *Bartonella muris* is thought to be latent in most rats, though the organism does not usually develop until the spleen is extirpated [Mayer, Borchardt and Kikuth, 1927]. Acute anaemia then results in a large proportion of the animals. Intact rats fed diets deficient in vitamins A and C may develop the disease [Wells and Mehta, 1930], and Meyer [1929] has shown that rats 3–4 weeks old are at the most susceptible stage and may be infected even when the diet is complete. He also found that the presence of *B. gärtner* sensitised some animals in a manner similar to splenectomy.

Newly-weaned rats (3–4 weeks old), restricted to a diet of cow's milk, consume only about 30 cc. daily, and thus receive only about 4 g. solid material per day compared with 10–15 g. consumed by rats on solid diets. The inadequate caloric value of the food coupled with the susceptible age of the rats [Meyer, 1929] give a set of conditions favourable to development of *Bartonella muris*.

It is likely, then, that the anaemia developed by milk feeding was partly due to *Bartonella* infection and such a possibility must be considered in other work, where milk diets have been employed for the production of anaemia. While it has been clearly demonstrated that copper feeding caused a more rapid regeneration of haemoglobin than did the feeding of iron alone, it was

[1] By kindness of Bacteriology Dept., Edinburgh University.

important to find if copper could exert any effect on haemoglobin production when the possibility of infection by *Bartonella muris* or *B. gärtner* was excluded.

A solid diet low in iron and copper was required, but the difficulty of compounding such a diet is illustrated by the results of analyses of foodstuffs (Table XII) likely to be free from these elements.

Table XII. *Iron and copper contents of some foodstuffs.*

Copper reported as mg. per kg. sample as purchased.
Iron reported as mg. per 100 g. sample as purchased.

Material	Copper	Iron	Material	Copper	Iron
Maize starch	0·8–1·9	3·18	"Casein" B.D.H. fat and vit.-free	3·9	—
Rice starch	0·8	2·02	Cheese (Scottish)	2·0	—
Potato starch	1·8	1·10	Cheese (Kraft)	0·45	1·23
Wheaten starch	2·4	0·7	Gelatin	9·6	—
Dextrin	2·2	—	Egg-albumin B.D.H.	1·4	1·00
Sago	Traces (0·1)	0·52	Egg-albumin (Chinese)	1·0	0·72
Castor sugar (sucrose)	Traces (0·1)	0·13	Butter (Scottish)	0·6	0·14
Arrowroot biscuits	1·5	1·03	Milk (Rowett Institute)	—	0·065 mg./100 cc.

By a selection from these materials it was, however, possible to devise a complete diet sufficiently low in iron and copper for use in an attempt to produce anaemia in growing rats.

Three groups of 6 rats were used in an experiment and the basal diet was as follows:

	Weight for 6 rats daily
Kraft cheese	3·0 g.
Egg-albumin	9·5 g.
Butter	9·5 g.
Sago	16·0 g.
Castor sugar	16·0 g.
Salt mixture	2·5 g.
Milk	60 cc.
Radiostol	1 drop

The salt mixture was McCollum's No. 185 modified by exclusion of ferric citrate and made up commercially from A.R. chemicals. Slight contamination had apparently occurred in commercial mixing since the salt mixture contained 2·2 mg. Fe per 100 g. Two further salt mixtures were prepared in the laboratory by the addition of purified "inorganic" iron to one and purified iron and copper sulphate to the other. These salt mixtures were used in the diets of two further groups of rats. The following amounts of iron and copper were supplied to the rats of the different groups.

	Intake per rat daily	
Group	Mg. iron	Mg. copper
I	0·058	0·006
II	0·35	0·006
III	0·35	0·046

The experiment lasted 18 weeks, haemoglobin determinations and reticulocyte counts being made at weekly intervals. The average haemoglobin values for the different groups are plotted in Fig. 3, while in all cases the percentage of reticulocytes was within the normal value of 2 % for the rat [Krumbhaar,

1930]. This normal value is in distinct contrast to the marked reticulocytosis of rats made anaemic by milk feeding and it is therefore unlikely that there was any complication from the infections referred to earlier.

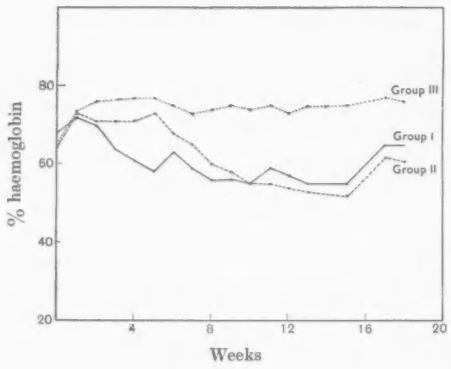

Fig. 3.

The results (Fig. 3) show that rats receiving no additions of iron or copper developed a mild anaemia which tended to disappear towards the end of the experiment. Supplementary feeding of inorganic iron had no effect in preventing the onset of this anaemia, but when copper was fed as well as iron the haemoglobin content remained normal throughout the entire period of the experiment.

Consideration of the weight increases shows that the effect of the copper is even more marked than is evident from the haemoglobin curves, for average growth in Group III was 76 g. while that in Group II was 57 g. and in Group I 55 g.

The additional copper, therefore, brought about the utilisation of iron for haemoglobin building, and inorganic iron was not used for this purpose unless copper also was available.

Other relations between copper and iron mètabolism.

In the first experiment in this connection, 4 rats which had been fed for 3 weeks after weaning on a diet of starch and cow's whole milk were housed individually in metabolism cages. Faeces and urine were collected separately and 5 days' excretion from each rat was made into one sample and analysed for copper. After two initial periods on a basal diet low in iron and copper, inorganic iron was fed daily to each rat. The effect of this iron feeding on copper excretion was determined.

To avoid metallic contamination, metabolism cages were constructed from 8-inch Büchner funnels. The animals were kept on the perforated plate and, to provide sufficient height, the sides were lengthened by perforated sheet zinc

wrapped round the outside where it was out of contact with the rat or with excreta. A glass plate covered the top of the cage. Faeces remained on the perforated plate of the Büchner funnel and were removed daily, urine passed through and was collected in a beaker placed beneath. Cages were washed out at end of each five-day period. The analytical results are given in Table XIII.

Table XIII.

Average of 4 rats.

Date	Diet	Mg. Cu in Faeces	Mg. Cu in Urine	Cu in faeces / Cu in urine
10–15. ii. 30	Basal	0·028	0·007	4·0
16–20. ii. 30		0·021	0·010	2·1
21–25. ii. 30	Basal + 0·5 mg. Fe per rat daily	0·018	0·004	4·5
26. ii. 30–2. iii. 30		0·017	0·007	2·4
3–7. iii. 30		0·018	0·007	2·6
8–12. iii. 30	Basal + 2·0 mg. Fe per rat daily	0·017	0·006	2·8
13–17. iii. 30		0·016	0·007	2·3
18–22. iii. 30	Basal	0·016	0·006	2·6
23–27. iii. 30		0·018	0·006	3·0
29. iii. 30–1. iv. 30		0·017	0·006	2·8

Before being used for experiment the rats were kept for three weeks from weaning time on a diet low in copper. This precaution was adopted so that stored copper obtained from sharing the mother's food would be eliminated. The results for the first period are probably affected by incomplete preparation of the rats. Omitting the first period from consideration it can be concluded that feeding inorganic iron did not alter the total amount of copper excreted or the proportion appearing in faeces and urine.

The average ratio $\frac{\text{copper in faeces}}{\text{copper in urine}}$ is 2·8 for a diet low in copper.

In another experiment two groups of rats were housed in glass cages similar to those already described and fed a basal ration of cow's whole milk *ad lib. plus* 3 g. sucrose per rat daily. One group was fed in addition 0·2 mg. purified inorganic iron per rat daily. At the end of 6 weeks, the animals were killed and copper determined in liver, heart, lungs, spleen, kidneys, brain, flesh, skin, tongue and testes. The data for this experiment are averaged in Table XIV.

Table XIV.

	Basal diet 11 rats	Basal diet + iron 10 rats
Average gain in weight during experiment	36 g.	37 g.
Total copper in parts analysed (calculated for one rat)	0·037 mg.	0·038 mg.

No significant differences between animals of the two groups were found in any of the organs or parts analysed. The results therefore indicate that addition of inorganic iron to a diet of milk *plus* sucrose has no effect on the copper content of the rat.

An experiment was designed to study the effect of feeding a diet almost free from copper on storage of iron in the foetus. Work which is discussed below (see Table XIX) showed that the iron content of rat pups from mothers

receiving a low ration of copper was slightly, though not significantly, less than that of pups from mothers receiving a normal ration of copper. That complete removal of copper from the diet cannot be effected by selection of foodstuffs is illustrated in Table XII. In the case of the rat, storage in the liver takes place after continued ingestion of even small traces of copper (Table IX), and between pregnancies the adult maternal animal is probably able to store sufficient copper to supply the needs of the foetal and suckling young. On the other hand, the hen produces an egg at regular short intervals and is therefore constantly depleting iron and copper reserves. It was decided, therefore, to use the hen for this experiment.

Four groups of 5 birds were used, the feeding being as follows:

Group I	Basal ration + iron	Capons
II	Basal ration + iron	Pullets
III	Basal ration + iron + copper	Capons
IV	Basal ration + iron + copper	Pullets

The basal ration was as follows, the daily amounts for 5 hens being given:

Kraft cheese	$\frac{1}{4}$ lb.
Purified maize starch	$\frac{1}{4}$ lb.
Castor sugar	$\frac{1}{4}$ lb.
Salt mixture	10 g.
Radiostol	5 drops

thoroughly mixed and fed without further treatment.

Also

Whole milk	1 pint
Granite grit	*ad lib.* (purest form of grit available)
Glass-distilled water	*ad lib.*
Granulated pure $CaCO_3$	*ad lib.*

Precipitated $CaCO_3$ was granulated by mixing with paste prepared from starch and drying at 100°.

Salt mixture I	Groups I and II	
	McCollum's salt mixture 185	21 parts
	Pure $CaCO_3$	9 parts
	Purified iron	0·03 part
Salt mixture II	Groups III and IV	
	0·003 part copper (as $CuSO_4, 5H_2O$) were added.	

In a preliminary experiment the diet for Groups I and II was shown to maintain weight and laying in a group of 5 hens over a period of 1 month.

The birds were confined inside on wooden floors covered with sawdust. Food was placed in wooden troughs and liquids in glass vessels. Trap nests were provided to obtain individual laying records. Feeding was started about one month before laying was expected to begin in order that first formation of eggs in the ovary should take place while the experimental diet was being consumed.

In all except two cases the experiment had been in progress for about 3 months before laying commenced.

Since reproduction makes a strong call on all reserves of the mother it was thought that the iron store in the liver might be depleted to fulfil the iron requirements of the egg. Two groups of capons were therefore included to act as controls for iron content of liver at the end of the experiment. These capons were procured from the same hatching of chickens as the pullets. The use of capons as non-laying controls for hens receives some justification from the facts of sex reversion in the fowl.

Results. Growth was similar in all groups and was rather slower than for normally fed hens. No significant differences in haemoglobin content of the blood occurred between any of the groups. When the experiment was concluded, 71 eggs had been laid by Group II hens and 69 by Group IV hens.

Iron estimations were made on the contents of all the eggs, composite samples being made from each hen with 2–4 eggs per sample. No significant differences were recorded and the averages of all eggs from each group are therefore given. Average analytical figures for twelve eggs from hens hatched at the same time as the experimental ones but fed a practical ration are included for comparison. Copper analyses are also included. These results appear in Table XV. In Table XVI are given the group averages of the results of analyses for iron and copper in the livers of all the birds at the end of the experiment.

Table XV. *Iron and copper content of eggs (averages).*

	% dry matter	Mg. Fe per egg	Mg. Cu per egg	% Fe on dry matter	Mg. Cu/kg. dry matter
Group II. Low copper	25·03	0·897	0·021	0·0087	2·0
Group IV. Fed copper	25·06	0·940	0·021	0·0085	1·7
Normal stock feeding	25·28	0·990	0·030	0·0090	2·2

Table XVI. *Iron and copper content of livers (averages).*

	Mg. Fe per liver	Mg. Cu per liver	% Fe on dry matter	Mg. Cu/kg. dry matter
Group I	18·64 Range 7·00–38·50	0·061	0·206	6·3
Group II	11·60 Range 8·50–16·58	0·057	0·110	5·7
Group III fed copper	9·87 Range 5·40–12·83	0·085	0·088	7·5
Group IV fed copper	14·05 Range 9·40–23·75	0·057	0·118	5·0

No significant differences were found between the iron and copper contents of eggs from Group II hens (each receiving about 0·07 mg. copper per day) and Group IV hens (each receiving 0·27 mg. copper per day). The amounts of iron in the livers of capons and of hens fed the experimental ration varied too much for any conclusions to be drawn.

Further experiments were conducted with rats to determine the effect of

copper feeding on the iron content of the livers and bodies of rats. The basal diet was cow's whole milk *ad lib. plus* 3 g. sucrose per rat daily, and the groups were treated as follows:

Group I Killed at weaning and analysed.
II Fed basal ration from weaning.
III Fed basal ration from weaning *plus* 0·2 mg. iron/rat daily.
IV Fed basal ration from weaning *plus* 0·2 mg. iron *plus* 0·05 mg. copper/rat daily.
V Fed basal ration from weaning *plus* 0·05 mg. copper per rat daily.

Feeding was continued for one month, after which the rats were killed and the iron determined in the liver, spleen, and rest of the rat (intestinal tract excluded). These conditions apply to the first two experiments; in the third experiment Group I was omitted and, in analysis, the spleen was included with the rest of the rat.

The average analytical results for each experiment are given in Table XVII.

Table XVII.

	Killed at weaning Group I		Basal diet Group II		Basal diet + Fe Group III		Basal + Fe + Cu Group IV		Basal + Cu Group V	
	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter
Exp. 1.	4 rats		2 rats		3 rats		2 rats		2 rats	
Liver	0·308	0·038	0·498	0·047	1·149	0·115	0·764	0·069	0·351	0·032
Spleen	0·066	0·090	0·119	0·090	0·251	0·166	0·272	0·195	0·185	0·109
Rest of rat	1·10	0·012	1·31	0·011	2·24	0·014	2·49	0·015	2·02	0·011
Exp. 2	4 rats		3 rats		4 rats		2 rats		3 rats	
Liver	0·249	0·053	0·302	0·037	0·716	0·079	0·845	0·086	0·320	0·038
Spleen	0·024	0·101	0·092	0·056	0·117	0·108	0·157	0·110	0·100	0·073
Rest of rat	0·89	0·015	1·01	0·007	1·65	0·010	1·79	0·017	1·30	0·011
Exp. 3			7 rats		4 rats		5 rats		3 rats	
Liver	—	—	0·615	0·056	0·663	0·046	0·650	0·055	0·597	0·043
Rest of rat	—	—	1·46	0·012	1·91	0·011	2·05	0·015	1·81	0·009

The results can be discussed from two aspects.

(*a*) *Effect of iron feeding.*

In Group II rats, which were fed a ration low in iron, the liver, spleen and body contain little more iron than do these organs from similar rats of Group I killed before commencement of feeding. Inclusion of iron in the ration of Group III has much increased the iron content, particularly in the case of liver. There is therefore some evidence to show that inorganic iron is stored in rats fed this element for a period of one month.

These remarks do not apply to the results of the third experiment, which provides no evidence of iron storage.

This disagreement regarding iron storage cannot be explained. A period of one month, however, is short and comparison of the iron content of liver from a

rat (single result) injected with 0·5 mg. iron daily for 6 months, with the content of liver from a normally fed stock rat illustrates the effect of continued dosage.

	Mg. Fe in liver	% Fe on dry matter
Rat injected	9·5	0·441
Rat normal	1·0	0·049

(*b*) *Effect of copper feeding.*

Comparison is made between Groups II and V and between Groups III and IV. In each case the only difference in the ration is the presence of copper. There is no evidence to show that copper increases the absorption or storage of iron. On the other hand, the results, while not perfectly clear cut, provide a balance of evidence to show that inclusion of copper in the diet has the effect of lowering the iron content of the liver and increasing that of the body. A possible interpretation of this is that copper promotes the utilisation of inorganic iron after it has been stored in the liver, and such an interpretation would agree with the results shown in Fig. 1, *F*.

The results just discussed together with those given in Fig. 1, *F*, showed that copper feeding probably caused utilisation of stored inorganic iron for haemoglobin building, and it was of interest to investigate this effect further. A method [Hill, 1930] became available for the determination in biological materials of "inorganic" iron as distinct from "organic" iron and some work was conducted to test the effect of copper feeding on the proportion of inorganic iron in the liver (Table XVIII). Hill has shown that *aa'*-dipyridyl, the reagent used in this method, does not react with reduced haematin, and the writer failed to obtain a test for iron with this reagent and the washed corpuscles of cat's blood. There are then grounds for the claim that the method will differentiate between "inorganic" and "organic" iron.

Table XVIII. *"Inorganic" and "organic" iron in livers of rats.*
(*Average values.*)

No. of rats, adults	Diet	"Inorganic" Fe		Total Fe		% Fe in "inorganic" state	% Fe in "organic" state (by difference)
		mg. Fe per liver	% on fresh matter	mg. Fe per liver	% on fresh matter		
10	Milk only	0·666	0·011	1·372	0·021	44·5	55·5
3	Milk + iron + copper	0·501	0·010	0·819	0·017	63·9	36·1
7	Milk + iron	0·650	0·011	1·403	0·023	45·2	54·8
2	Milk + manganese	0·222	0·004	0·476	0·008	46·6	53·4
2	Milk + cobalt	0·443	0·011	0·716	0·017	61·8	38·2
6	Milk + copper	0·204	0·004	0·614	0·012	28·6	71·4
3	Copper fed for 1 month Milk + copper Copper fed for 2 months	0·054	0·0008	0·361	0·005	15·2	84·8
Newly born rats	Mothers' diet						
8	Normal copper content	0·067	0·019	0·116	0·033	57·3	42·7
22	100 times normal copper content	0·034	0·013	0·077	0·030	42·0	58·0

These results indicate that the highest percentage of "organic" iron in terms of total iron was obtained in the cases where copper alone was the supplement to a milk diet: this percentage was increased by prolonged feeding

and was accompanied by a definite diminution in the total iron content of the liver. Where "inorganic" iron was added as a supplement along with the copper, there was no increase in the proportion of "organic" to total iron, probably owing to a continual addition to the stores of "inorganic" iron in the liver. Further it was observed that a high proportion of copper in the mothers' diets had a slight tendency to increase the proportion of "organic" iron in the livers of newly born rats.

No similar effects were observed when cobalt or manganese were fed as supplements without iron.

The mechanism of the action of copper in promoting haemoglobin building.

The effect of copper in promoting the utilisation of inorganic iron in haemoglobin building has been confirmed and it is of importance to consider the mechanism of this action.

The chief activity of copper is probably located in the liver, since, at all stages of growth of the animal, this organ is by far the richest in that element, and since, also, it has been shown that copper feeding causes the utilisation of iron stored in the liver for haemoglobin production (Fig. 1, *F*, and Table XVII).

Further, it has been shown, in the case of rats, that copper feeding definitely increases the proportion of "organic" to total iron in the liver, and this change from "inorganic" to "organic" iron is probably a step in haemoglobin building dependent on the presence of copper. Any modification of the state of the iron would be expected to be in the direction towards the condition of iron in haemoglobin, and it is therefore possible that copper may promote the change from inorganic iron to an iron porphyrin. Elvehjem [1931] found that the addition of copper to the nutrient medium of baker's yeast caused the formation of cytochrome *a* from inorganic iron, and if a similar process goes on in the rat some indication would be given that haemoglobin is derived from a haemochromogen.

It is of interest to enquire into the process whereby copper may bring about the formation of an iron porphyrin, though the suggestion to be made is, of course, entirely speculative.

(*a*) Turacin, the pigment of certain South African birds, is a naturally occurring copper porphyrin, and by chemical manipulation the copper can be displaced by iron giving a compound practically indistinguishable from haematin [Laidlaw, 1904].

(*b*) It was observed by Warburg and Krebs [1927] that after bleeding, *i.e.* when blood regeneration is rapid, the percentage of loosely bound copper in blood-serum is increased, while the percentage of loosely bound iron is decreased.

It is possible therefore, that the effect of copper in increasing the proportion of "organic" iron is achieved by a preliminary formation of a copper porphyrin and subsequent replacement of copper by iron. This would fit in with the observations outlined above and with the general chemical nature of the elements. The fact that injection of haematoporphyrin or protoporphyrin did

not increase blood regeneration (Fig. 1, *I*) indicates that the formation of the porphyrin unit itself is not the function of copper.

III. Toxicity of copper to animals.

It is now fairly generally agreed by writers on toxicology that acute poisoning by copper, resulting in death, is rare, since toxic doses are so large that they are immediately rejected by the stomach.

The position in respect to chronic copper poisoning is not so clear and whilst Pecholier and St Pierre [1856] stated that slow absorption of copper was advantageous, Mallory [1925] has expressed the view that continued exposure to copper, such as is experienced by workers in copper foundries, will cause haematochromatosis. In support of Mallory, Funk and St Clair [1930] have reported a case of haematochromatosis in man where the proportion of copper in the liver was extremely high.

Feeding experiments with animals have been conducted chiefly with rabbits, the procedure being to feed copper acetate for a period and to observe the extent of pigmentation in the livers. No detailed observations regarding growth, fecundity *etc.* of animals receiving an abnormally high ration of copper have yet appeared, and experiments on this aspect have therefore been conducted with rats.

The experiments included, in all, six groups—the same basal ration being used throughout but with differing amounts of copper salts included in the salt mixture for Groups II–VI.

Details of the groups were as follows:

Group I. 14 newly weaned rats, 9 females and 5 males. Basal ration 0·011 mg. copper per rat daily.

Group II. 14 newly weaned rats, 9 females and 5 males. Copper sulphate added to give 0·050 mg. copper per rat daily.

Group III. 8 newly weaned rats, 5 females and 3 males. Copper sulphate added to give 7·5 mg. copper per rat daily.

Group IV. 5 adults, 3 females, 2 males. Copper sulphate added to give 7·5 mg. copper per rat daily.

Group V. 5 adults, 3 females, 2 males. Copper acetate added to give 7·5 mg. copper per rat daily.

Group VI. 5 adults, 3 females, 2 males. Basic copper acetate (verdigris) added to give 7·5 mg. copper per rat daily.

The basal ration low in copper was as follows:

Kraft cheese	45 g.
Butter	7·5 g.
Maize starch	65 g.
Castor sugar	35 g.
Salt mixture I	5 g.
Radiostol	3 drops
Milk	150 cc.

This weight of food was sufficient for fifteen rats.

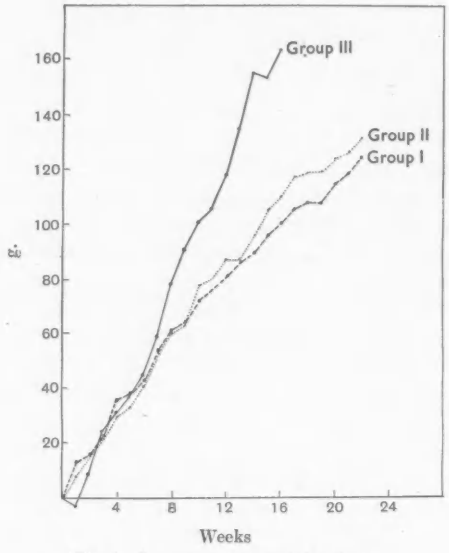

Fig. 4. Average gain in weight of rats.

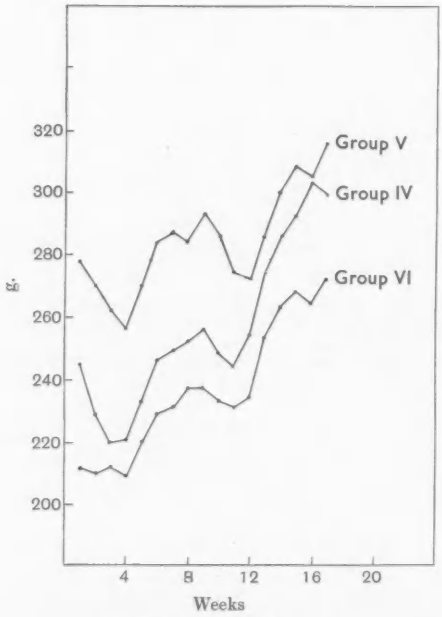

Fig. 5. Average weight of rats.

The rats were weighed weekly. For Groups I–III the average gains for one rat at weekly intervals are plotted in Fig. 4, although the growth curve for Group III is not strictly comparable with curves for Groups I and II since the former group was started at a different time of the year. In Fig. 5 are plotted the average weights per rat for each of groups IV–VI.

Haemoglobin determinations were made at irregular intervals on all rats and normal values were found without exception.

Table XIX contains the reproduction records of rats from each group. Some of the rat pups were killed at birth and analysed for copper and iron; the results are also included. The remaining young were reared successfully in all cases.

Table XIX.

Group	I	II	III	IV	V	VI	Stock diet; 1 litter for comparison.
Copper in diet per rat daily:	0·011 mg. $CuSO_4$	0·050 mg. $CuSO_4$	7·5 mg. $CuSO_4$	7·5 mg. $CuSO_4$	7·5 mg. Cu acetate	7·5 mg. verdigris	About 0·05 mg. Cu
No. of females in group	8	8	5	3	3	3	—
No. of litters	8	9	4	3	3	Nil	—
No. of pups per litter (av.)	6·9	7·9	6·3	8·0	6·7	—	8
Av. wt. of one pup at birth (g.)	4·8	6·6	6·0	5·7	4·8	—	5·0
Total number analysed	24	21	Nil	13	7	—	8
Mg. Fe/rat pup (av.)	0·268	0·312	—	0·352	0·316	—	0·275
Mg. Cu/rat pup (av.)	0·0045	0·0073	—	0·0145	0·0147	—	0·0046
% Fe on dry matter (av.)	0·0374	0·0320	—	0·0412	0·0467	—	0·0354
Mg. Cu/kg. dry matter (av.)	6·3	8·0	—	16·2	20·9	—	5·9

The following conclusions may be drawn from this experiment.

(1) The first generation of rats throve on a diet providing as little as 0·011 mg. copper to each rat daily. In comparison with rats receiving a normal amount of 0·05 mg. copper daily the low-copper rats grew at a slightly slower rate, the number of pups produced in one litter was slightly less and the average weight of individual rat pups was somewhat smaller. Differences were not marked and sufficient data are not available to decide whether these are significant. At the same time, since the ration proved to be a good one, the low-copper rats grew rather faster than is normally the case for the stock colony, and their haemoglobin was maintained at a normal level.

(2) Analyses of the young produced by these two groups of rats showed on the average no marked difference in iron content but the copper content was significantly lower in those pups whose mothers were fed a low ration of copper. It will be seen that there is a further increase in the copper content of young rats from breeding mothers receiving 7·5 mg. copper per day. There are then,

some indications that the copper content of the diet of rats will affect the storage of copper in the foetus. Hart *et al.* [1930] have shown that the copper content of the diet of sows has no effect on the copper transmitted to the young pigs. These differences between the two species perhaps have some bearing on the facts that while copper promotes the utilisation of inorganic iron for haemoglobin building in the rat it has been claimed that in the pig [Hart *et al.*, 1930] it is not necessary for this purpose.

(3) The presence in the diet of over 100 times the normal amount of copper (as copper sulphate) has no detrimental effect on the growth of rats. Normal young may also be produced on such a ration.

(4) The presence in the diet of over 100 times the normal amount of copper as copper acetate or as verdigris was not toxic to adult rats. Again, histological examination of livers after 5 months' feeding did not reveal any deposition of pigment. Breeding was normal in those rats fed copper acetate but no litters were produced in the same time by the animals receiving verdigris.

Summary.

1. Determinations of copper have been made in plants, foodstuffs, insects and marine animals. A systematic survey has also been made of the occurrence of copper in the organs of twelve species of land animals (foetal, newly born and adult).

2. The copper content of the skin and of the livers of rats has been shown to be increased in relatively small degree compared with the additional copper fed in the diet. Feeding additional copper to the mother caused increased storage of copper in the newly born rat.

3. A catalytic effect of copper on the oxidation of "dopa" to melanin has been observed. A suggestion has also been made that the copper in the ink sac of the octopus plays some part in the formation of the ink.

4. Copper feeding has been shown to have little or no effect on the storage of iron in the foetal rat or in the hen's egg.

5. Iron feeding has been shown to have no effect on the storage or excretion of copper by the rat.

6. The use of milk diets for production of a nutritional anaemia in rats has been criticised. At the same time the stimulating effect of copper on the formation of haemoglobin in rats has been confirmed and a possible mechanism suggested for its action.

7. It has been shown that feeding high amounts of copper sulphate or copper acetate has no effect on the growth and reproduction of rats, while high amounts of verdigris were observed to stop reproduction. It has also been shown that rats receiving one-fifth of the normal amount of copper in the diet will grow and reproduce normally.

The work reported above was carried out during a visit of the writer from New Zealand. This visit put into effect a scheme for the interchange of workers

in Agricultural Science, proposed at the Imperial Agricultural Conference in 1927. I desire to express my appreciation of the opportunity thus afforded, of carrying out research work in this country.

I also wish to express my very great indebtedness to Mr W. Godden for his continued interest and for his counsel throughout the investigation, and to Dr J. P. McGowan for his advice on problems of pathology and haematology.

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CXLII. ON THE FUNDAMENTAL NATURE OF VITAMIN D ACTION.

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(Preliminary Communication.)

From the Rowett Research Institute, Aberdeen.

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In a previous paper [McGowan, 1930] it was shown that, in chloroform poisoning in the rabbit, there is an accumulation of fatty substances together with calcium in certain of the tissues, especially the liver. This was interpreted as meaning that chloroform attacked and broke up the lipins, thereby setting phosphoric acid free to be neutralised *in situ* by calcium to form insoluble calcium phosphate. A similar deposition of calcium salts in certain of the tissues, following on a preliminary degeneration, has been recorded as the main feature of vitamin D poisoning [Duguid, 1930; and others]. Experiments were therefore undertaken to see if this hypervitaminosis D was explicable on a similar basis.

Rabbits were employed. These, for several weeks prior to the experiment, had been kept on the experimental ration, which was a modification of the stock ration in the direction of a considerable increase of its calcium carbonate content. The stock ration consisted of bran 8 parts, white sharps 8, bruised oats 8, and fish meal 1. To this was added, to form the experimental ration, 2 parts calcium carbonate, making thus roughly 8 %.

The rations were fed *ad lib.* and, in every case, in addition there was an abundant supply of freshly cut clover-rich grass. Water, for drinking purposes, was always available.

The vitamin D employed was in the form of a solution of irradiated ergosterol in olive oil, of a strength of 1,000,000 rat units per cc. For a supply of this we are greatly indebted to Mr A. L. Bacharach of the Glaxo Research Laboratories. It was administered by mouth by means of a pipette twice daily. So far the dosage employed has been 4–8 million rat units per day.

In Table I are summarised some results obtained in illustrative cases.

Table I deals with results obtained from a rabbit (No. 2) poisoned with vitamin D; a rabbit (No. 4) poisoned with chloroform; normal rabbits (Nos. 5, 10 and 11), fed on the stock ration for the composition of normal urine for comparison purposes; and two normal rabbits for the calcium content of the liver.

Table I.

Rabbit and dose	Diet	Date	Weight g. A	Cc. urine in 24 hrs. B	Nature of deposit, *etc.* — Crystals C	Gas with acid D	Urine reaction C	Total CaO as carbonate % E	Total CaO % F	Total P_2O_5 % G	CaO in 24 hrs. g. H	P_2O_5 in 24 hrs. g. I	Ca in blood mg./100 cc. J	P in blood mg./100 cc. K	Ca % moist weight liver L	Ca % moist weight kidney M
No. 2. Irradiated ergosterol, 4 million rat units daily commencing 11. vi. 31	Stock ration +8 % $CaCO_3$ +grass, all *ad lib.*	10. vi. 31	2020	72	Almost pure $CaCO_3$	+ + + + + +	Alk.	—	0·36	0·01	0·259	0·007	17·5	3·03	—	—
		11. vi. 31	2030	150	do.	+ + + + + +	do.	—	0·44	0·01	0·660	0·015	—	—	—	—
		12. vi. 31	1950	121	Almost pure amorphous phosphates	+	do.	—	0·64	0·13	0·774	0·157	—	—	—	—
		13. vi. 31	1950	65	do.	+	do.	—	0·57	0·46	0·370	0·299	—	—	—	—
		14. vi. 31	1840	72	do.	+	do.	0·13	0·42	0·47	0·302	0·338	—	—	—	—
		15. vi. 31	1795	85	do.	+	do.	0·04	0·50	0·46	0·425	0·391	—	—	—	—
		16. vi. 31	1750	*												
		16. vi. 31	Died	22·5†	do.	+	do.	0·17	0·64	0·29	—	—	—	—	0·0076	0·23
No. 4. 1·0 cc. $CHCl_3$ on 17. vi. 31 subcutaneously	Stock ration +8 % $CaCO_3$ +grass, all *ad lib.*	15. vi. 31	1590	—	—	—	—	—	—	—	—	—	—	—	—	—
		16. vi. 31	1650	274	Almost pure $CaCO_3$	+ + + + + +	Alk.	0·60	0·61	0·02	1·671	0·055	15·3	5·3	—	—
		17. vi. 31	1640	145	do.	+ + + + + +	do.	0·95	1·17	0·03	1·697	0·044	—	—	—	—
		18. vi. 31	1600	82	Almost pure amorphous phosphates	+?	Neutral	0·00	0·33	0·43	0·271	0·353	13·8	3·2	—	—
		19. vi. 31	1550	144	do.	+	Alk.	0·03	0·15	0·08	0·216	0·115	—	—	—	—
		19. vi. 31	Killed	25†	do.	+	do.	0·05	0·14	0·05	—	—	—	—	0·0078	0·028
Normal. No. 5	Stock ration alone + grass	20. vi. 31	1800	130	None	None	Acid	None	0·04	0·26	0·052	0·338	14·8	3·2	—	—
No. 10		20. vi. 31	2350	115	do.	do.	do.	do.	0·009	0·46	0·010	0·520	—	—	—	—
No. 11		20. vi. 31	1900	50	do.	do.	do.	do.	0·039	0·18	0·078	0·360	—	—	—	—
Normal rabbit	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·0065	—
Normal rabbit	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·0066	—

* Urine lost by being passed into food dish. † In bladder.

Column A shows the loss of weight from vitamin D and chloroform poisoning. Rabbit 2 ceased to eat on the 14th, two days after vitamin D administration commenced, whereas rabbit 4 stopped on the 17th, as soon as the chloroform had been administered. These occurrences have to be considered in relation to the results obtained below.

Column B gives the amount of urine passed in 24 hours. When the animal died or was killed, the urine present in the bladder was also recovered.

Column C deals with the reaction and the nature of the deposit present in the urine as observed microscopically. In the rabbits on high-calcium diet, the urine was alkaline; on stock ration, acid. In rabbits 2 and 4 on high-calcium diet, the deposit prior to poisoning consisted practically entirely of calcium carbonate crystals. Immediately on poisoning, the calcium carbonate was replaced by amorphous phosphates. There was no deposit present in the normal rabbits on stock ration.

In Column D is given the effect of adding acid to the microscopical preparations. For comparative purposes, a record in arbitrary symbols is given of the amount of gas (CO_2) evolved. It will be seen that this supports the findings in Column C.

In Column E is recorded, as CaO % in the urine, the amount of calcium present as carbonate, as calculated from the amount of gas collected on making the urine distinctly acid. This line of observation was not adopted till 14. vi. 31, hence the results for rabbit 2 prior to this date are lacking. From those obtained in Column D, however, they are undoubtedly quantitatively much the same as in rabbit 4 for the pre-poisoning period. The great diminution of the calcium in the form of carbonate in the poisoning period is again evident, while the very great excess of calcium as carbonate or phosphate in the urine of the experimental animals over that in the urine of the normal rabbits on the stock ration is demonstrated.

Columns F and G give respectively the percentage total CaO and P_2O_5 in the urines. These results, however, can only be discussed in relation to Columns H and I.

In regard to Column H, it will be seen that in rabbit 2 the calcium in the poisoning period remains more or less at the level it was in the pre-poisoning period, with the exception of a possible fall at the very end (see Column F), whilst in rabbit 4 there is a very marked drop in the poisoning period. The result in rabbit 4 may be connected with the diminished intake of food, with its high calcium content, which occurred during this period.

In Column I is demonstrated the very marked increase in the poisoning period of the P_2O_5, more gradual in the case of rabbit 2, with a definite falling off in rabbit 4 and a possible one also in rabbit 2 towards the end of the period. This terminal fall may possibly be attributed to metabolism as a whole having more or less ceased, owing to the animal being *in extremis*, or to the supply of readily available lipins, from which phosphorus could be split off, being used up. The results, therefore, as far as regards the phosphorus, indicate a greatly

increased excretion of phosphorus, which agrees with Duguid's summing up of the situation [1930], where he states that "there is a very greatly increased excretion of phosphorus especially by way of the kidneys and this, occurring when the food consumption is diminishing as is usual in hypervitaminosis D, involves a reduction in the phosphorus retention in the body." Duguid [1930] also states that an increased excretion of calcium in the urine is the rule in such cases. The different result obtained in the present instance may have reference to the very high calcium content of the food and the high level of calcium excretion in the urine to begin with.

The results in Columns E, H and I go to show that the calcium, formerly excreted as carbonate, is got rid of in the poisoning period in the form of phosphate.

The bones of the rabbits were ivory hard and showed no trace of thinning of the cortical bone or of any demineralisation process.

In Columns J and K a few results are given of the estimation of Ca and P in the circulating blood. This mode of observation, however, was not pressed. It was recognised that the withdrawal of 10 cc. of blood from a rabbit at frequent intervals would jeopardise more important results, while it was also borne in mind that, in the poisoning stage, owing to the depressed condition of the circulation, it is often impossible to obtain a sample of blood. More important still, it was realised that, in such procedures, one is estimating the Ca and P in a sample of blood taken in its passage from an irregular and varying "source" or "sources" to an irregular and varying "distribution," two variables regarding the essential activity of either of which, at the time the blood sample was being taken, there is and can be, under present circumstances, no real knowledge. Thus, while a high Ca % in the blood might mean a high intake from the "sources," it might equally well denote a diminished "distribution." A similar state of matters exists in regard to phosphorus. There is also possible a variety of further permutations and combinations of "source" and "distribution" with an effect on the blood composition.

In a previous communication [McGowan, 1930, Table, p. 288], it was shown that in chloroform poisoning there was a great increase in the amount of fat and calcium salts in the central two-thirds of the liver lobules. The amounts present of these two substances ran more or less parallel. Where relatively small amounts of each existed, the histological picture showed that these were remnants, left from large amounts previously present, which, in the meantime, had been removed by the circulation. This process of removal is a rapid one, for the experiments lasted in most cases for a few days only. In these experiments also, the fatty material was present not only as neutral fat but to a large extent as calcium soap. For comparison with the figures obtained in the present investigation, it may be stated that, in one of these cases of chloroform poisoning in the rabbit, the calcium as metal present in the liver was as much as 2·66 % of the moist weight. On the other hand, figures as low as 0·025 % were obtained.

In the present case of chloroform poisoning (rabbit 4) the findings were quite different from those just stated. The inner two-thirds of each lobule was indeed crammed with lipoid material, which, on staining with Nile blue sulphate, was found to be neutral fat without any fatty acids. Von Kossa's silver nitrate test for calcium phosphate gave entirely negative results, while the amount of calcium present was only 0·0078 % of the moist weight, a figure differing but little from the normal (*vide* bottom of column L). It is difficult to explain the anomalous condition present here unless one assumes that the large amount of $CaCO_3$ in the diet had prevented the absorption of inorganic phosphates from the food, thus rendering the blood relatively deficient in, and avid for, inorganic phosphate and so withdrawing the latter from the tissues whenever and as soon as it was formed [*cf.* Cushny, 1918].

If one now turns to consider the appearances present in the livers of the rabbits poisoned with vitamin D, frozen sections stained with Sudan III gave no evidence of the presence of fatty material in any considerable amount. In this connection, however, it should be noted that Haendel and Malet [1930] have found large quantities of fat in the liver, kidney, heart and spleen of rabbits poisoned with vitamin D. Von Kossa's stain also gave a negative result with regard to the presence of calcium phosphate. Fat, nevertheless, may have been present in the cells of the present cases in globules too minute to be detected by the coarse technique just alluded to. Osmic acid preparations have not as yet been made, but ordinary paraffin sections show a very advanced stage of cloudy swelling, with granular degeneration and a basophil reaction of the cytoplasm, conditions found present also in the liver cells at the periphery of the liver lobules in chloroform poisoning. This latter appearance may indicate a reaction to the acid side, which may possibly be due to acid phosphates, derived from a breaking down of the lipoids. In any case, cloudy swelling is the well-known precursor of the ordinary easily recognisable fatty degeneration. The calcium present varied little from normal, being 0·0076 % of the moist weight.

The histological changes in the various organs and tissues of the rabbits poisoned with vitamin D may now be discussed. They were practically those found by previous observers. The following tissues were examined: liver, spleen, kidney, testicle, suprarenal, heart muscle, wall of aorta, lung, skeletal muscle, stomach wall, small intestine, colon and bone marrow. Briefly put, the changes observed were, in the first place, cloudy swelling and granular degeneration, especially present in the liver cells, secretory tubule cells of the kidney and in the muscle of the heart, arteries and stomach wall. Deposition of calcium salts was found present in the media of the arterioles of the cortex of the kidney and of the lung and in the media of the aorta. Specially, there was no deposition in the heart muscle or in the arterioles of the heart. There were large patches of precipitation in the muscular wall of the stomach of such size that they could be seen by the naked eye as white striations. A few small spots were also present in the gastric mucosa. As already suggested in relation

to rabbit 4, the high calcium diet may have interfered somewhat with calcium deposition. The kidney showed the presence of calcium in its tubules. Doubtless this is to be explained by the high excretion of calcium in the urine and is not necessarily indicative of a degenerative change in the epithelium. The relatively high calcium in the kidney (*vide* Column M) seems also explicable on this basis, the higher figure for rabbit 2 being an index of the increased calcium in the walls of the arterioles.

The suprarenals in these cases were much enlarged, the diameter being about five times that of the normal gland. The enlargement was mainly in the cortex at the expense of the medulla. The cortex was pure white in colour and gave a marked Sudan III reaction, while in paraffin sections the cytoplasm of the cells stained deeply basophil. The significance of these findings awaits elucidation.

Contrasting the condition found in these cases with that present in chloroform poisoning, it would seem that, in both instances, there is an attack on the protoplasm of the cell. In chloroform poisoning, however, there is a much larger amount of the poison active at any one time, the condition being, in every respect, a more fulminating one. The poisoning produces its maximum effect almost at once, to be followed almost immediately by phenomena of recovery. Hence the lipins are broken up rapidly, fatty bodies and calcium phosphate being produced in large abundance *in situ* without time being given for their removal into the circulation. Thus, within the time employed for such investigations, in most instances they remain to some degree in the tissues and are observed on histological examination. Evidence has, however, already been adduced to show that they can be, and are at times, fairly rapidly removed [McGowan, 1930, Table, p. 288].

In the case of vitamin D poisoning, however, the amount of poison active at any one time is very small, the attack on the cell and its lipins is not so drastic, and ample time is given for the removal of by-products. Hence, on histological examination, the appearances found are much milder in type and calcium is found only in those situations, the media of the arterioles, *etc.*, where, once having been deposited, it cannot so readily be removed as it can in the case of parenchymatous cells of actively metabolising glands, like the kidney and liver, which abut on a rich blood supply. This view is supported by the results obtained by Hertzenberger [1929], who found evidence of the healing of the lesions and disappearance of the calcium deposits after cessation of administration of vitamin D and by those of Schiff [1930], who found no evidence of resorption of the calcium from the arterial lesions after poisoning was stopped.

The rather insignificant pathological findings just described have been obtained in the case of an animal which actually died within 5 days from the effects of the poisoning. It is a question therefore whether changes of this degree are not the essential ones and whether the more advanced changes, involving parenchymatous cells of various organs in fatty change and calcium

deposition, which have been described, may not have to be attributed to an anoxaemia brought about by the alterations in the vascular walls.

The broad aspects of the subject in hand may be discussed and the position summed up by a consideration of some points raised by Cushny [1918] relative to phosphorus and calcium metabolism. On p. 108, he makes the statement that in the herbivora phosphates are excreted exclusively by the bowel wall. Obviously, this generalisation is incorrect, for, in Table I, it will be seen that rabbits 5, 10 and 11, on the stock ration, pass considerable quantities of phosphates in the urine. Dealing with the subject of calcium metabolism at p. 560, Cushny states that calcium is excreted in part by the urine but for the most part through the epithelium of the large intestine. The relative amounts excreted by the kidney and bowel seem to be determined by the quantity of available phosphates among other factors. If these are present in large quantities in the blood, the calcium is excreted mainly in the bowel in the form of calcium phosphate. The elimination of calcium thus appears to vary with the character of the combinations which it can form. If these are soluble they appear in the urine, while the insoluble ones tend to pass into the stools. Calcium lessens the phosphates of the urine by forming insoluble phosphates in the bowel and thus preventing the absorption of the phosphates of the food.

It seems possible to harmonise these views of Cushny with the results obtained in our experiments. It may be supposed that, in the rabbits fed on the stock ration, there is not enough calcium to join up with the phosphate and carry it in the insoluble form of calcium phosphate to the bowel for elimination. There is an over-plus of phosphate which appears in the urine as alkaline phosphates. With more calcium in the food, it could be supposed that practically all the phosphate goes as calcium phosphate to the bowel, leaving only a trace to appear in the urine. *A fortiori*, this holds for conditions where there is great excess of calcium in the diet, with the further significant occurrence that the excess calcium appears in the urine as the carbonate conveying CO_2 out of the body, a function more efficiently performed by the lungs. In conditions of vitamin D and chloroform poisoning in animals fed excess of calcium carbonate, there is great excess of phosphate produced, more than can be got rid of as calcium phosphate by the intestine. The excess calcium phosphate produced now goes by the kidney, the calcium formerly excreted as carbonate being utilised for this purpose. One slight blemish—it is, however, a slight and unimportant one from the present point of view—appears at first sight to mar the complete correlation. Cushny states that the insoluble calcium compounds pass by the bowel and apparently by the bowel only. Yet, it is indubitable that, in the present experiments, not only has calcium phosphate passed by the kidney (for this of course an explanation has been offered), but also such an insoluble substance as calcium carbonate. Doubtless, however, Cushny's statement will hold if the food supplies no more calcium than can be dealt with by the phosphate in the blood and so be removed as insoluble calcium phosphate by the intestine.

It seems possible that the findings of Harris and Innes [1931] that vitamin D poisoning is accentuated by increase of calcium in the diet, but that vitamin D, even in large doses over a long period, becomes relatively harmless when calcium is balanced by the addition of phosphate or when calcium is omitted and phosphate remains high, may derive some explanation from what has just been discussed. For one thing, there will be less calcium present to form deposits and, for another, the less speedy "insoluble" method of elimination by the bowel in the form of calcium phosphate may be supplanted by the more speedy "soluble" method of elimination by means of the kidney in the form of acid phosphates of the alkali metals.

The subject remains for discussion as to the relation of the present findings to the question of the nature of rickets and of the action of vitamin D in this disease. Elsewhere [McGowan, 1926], it has been pointed out (and the implications emphasised) that rickets begins very early during the suckling stage of the animal. Evidence was also adduced to show that the appearance of rickets depends on a deficiency of consolidating materials in the milk relative to the growth-promoting ones and to the growth potential of the young animal. Further, it has been shown that the administration of inorganic phosphate, in the form of sodium phosphate, can prevent and cure rickets; in the fowl, by Bethke, Kennard, Kick and Zinzalian [1927–28] and in the rat by Karelitz and Shohl [1927] and Kramer, Shear and Siegel [1931]. From our previous discussion and these considerations, we draw the conclusion that there is more to be said for a relative deficiency of inorganic phosphate being the actual *causa causans* of rickets than for the lack of any other substance hitherto alleged to be operative[1].

As to the possible mode of action of vitamin D in combating the disease, apart from what has already been adumbrated above in this direction, an insight may be gained by a consideration of the significance of the persistent low blood phosphorus that occurs in rickets. This low phosphorus may be attributed, on the one hand, to the constant removal, for constructional purposes, from the blood of inorganic phosphorus by the avid bony tissues and, on the other hand, to a low supply to the blood from the diet which is deficient in inorganic phosphorus. This can be rectified, directly, by supplying additional inorganic phosphorus in the diet or, indirectly, by supplying a substance—vitamin D—which will make available to the blood the inorganic phosphorus stored in the body lipins. Further, it seems obvious that the supply of great excess of inorganic calcium in the diet, by its action in rendering the inorganic phosphates of the diet insoluble and unabsorbable, will predispose to the production of rickets.

[1] A calcium-deficient ration produces osteoporosis, not rickets. At the same time, it should be noted that rickets is not, in our opinion, due essentially and primarily to a lack of vitamin D.

SUMMARY.

Evidence has been adduced to show that the probable mode of action of vitamin D in the cure and prevention of rickets is by the setting free from the lipins of the body of inorganic phosphate, the relative deficiency of which is regarded as the essential cause of rickets.

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CXLIII. STUDIES ON HEMICELLULOSES.
IV. THE PROXIMATE ANALYSIS OF BOX-WOOD, AND THE NATURE OF ITS FURFURALDEHYDE-YIELDING CONSTITUENTS.

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Introduction.

As a preliminary to an investigation of the nature of the hemicelluloses of box-wood, it was desired to obtain as complete an idea as possible of the constitution of the wood, and with the minimum of overlapping among the constituents estimated.

Preliminary experiments had indicated that the furfuraldehyde-yielding constituents of the wood could be separated into a number of groups. Thus, hot 0·5 % ammonium oxalate solution removed from the sawdust both pectin and a small amount of non-pectic substance capable of yielding furfuraldehyde. Again, extraction of the residue from this treatment with boiling alcoholic sodium hydroxide solution dissolved in addition to lignin a substance which was reprecipitated on rendering the extract acid. This product, here described as "lignosaccharide," appeared to resemble the hemicelluloses closely in properties and constitution. Extraction with 4 % sodium hydroxide solution at room temperature removed "free hemicelluloses," but a portion of the hemicellulosic substance still remaining in association with the tissue residue required more drastic treatment for its removal. This "combined hemicellulose" was dissolved out by hot 4 % sodium hydroxide solution, but an amount of "other pentosans" remained undissolved, and this was shown to consist largely, if not entirely, of xylan.

Means were found for estimating separately all the above groups with the exception of the non-pectic substance soluble in ammonium oxalate solution. This substance was also found to be soluble in hot water and is included in the results of analysis under the heading "hot water-soluble." In addition, a number of other determinations were made, including ash, alcohol-ether-soluble substances, protein, lignin and cellulose, and it is thought that no constituent of the wood present in significant amount escapes inclusion under these headings.

EXPERIMENTAL.

A. Proximate analysis.

Methods of analysis.

Furfuraldehyde-yielding constituents. The estimation of hemicelluloses is a matter of some difficulty. The alkali-soluble "furfuroids" of Cross, Bevan and Smith [1895] would correspond roughly to hemicelluloses, but the figures obtained by these authors can only be approximations. Konig and Becker [1919] determined hemicelluloses in wood by heating with 0·4 % sulphuric acid at arbitrary pressures, the sugars arising being estimated in the resultant hydrolysis liquid. Dore [1920] determined the loss in weight of woods on extraction with 5 % NaOH. Other workers have distinguished "pentosans in cellulose" and "pentosans not in cellulose."

A method for the determination of hemicelluloses in cereal straws has recently been described by Norman [1929], it being assumed that the only furfuraldehyde-yielding constituents of the straws are pectin, free hemicelluloses and xylan associated with cellulose. Attempting to apply this method of estimation to box-wood, the following figures were obtained.

Table I.

Total furfuraldehyde on dry wood	11·86 %
Calcium pectate yield	0·41
Cellulose (Cross and Bevan)	49·35
Furfuraldehyde yield % of cellulose (Cross and Bevan)	10·35
% xylan in cellulose	16·04
% xylan calculated to dry wood	7·92

It may be pointed out that a knowledge of the carbon dioxide yield of wood and hemicellulose, obtained by the method of Nanji, Paton and Ling [1925], is not necessary to the calculation.

100 parts calcium pectate yield 19·5 parts furfuraldehyde.
100 parts xylan yield 64·5 parts furfuraldehyde.

Hence, furfuraldehyde arising from hemicellulose is given by

$$11{\cdot}86 - \{(0{\cdot}41 \times 0{\cdot}195) + (7{\cdot}92 \times 0{\cdot}645)\} = 6{\cdot}67\ \%.$$

The yield of furfuraldehyde from the hemicellulose being 55·14 %, the percentage of hemicellulose on the dry wood should be given by the quotient of 6·67 and 0·5514, *i.e.* 12·09 %. However, carefully carried out preparations of the hemicellulose mixture gave yields amounting to no more than two-thirds of this value, and similar discrepancies were found with other woods. It is therefore apparent that this method of determination is not applicable to woods.

It was decided, therefore, in the present work to determine the hemicelluloses by actual preparation, by a method which is in essentials that described by Norris and Preece [1930].

20–25 g. of the wood are extracted on the boiling water-bath with 300 cc. of 0·5 % ammonium oxalate solution for 1½ hours, the mass after cooling being filtered on paper at the pump. The residue is washed back into the extraction flask and extraction with ammonium oxalate is repeated. The filtered residue is then treated for 1½ hours with 300 cc. of boiling 50 % alcohol containing 1 % of sodium hydroxide. After further filtration through paper at the pump, the residue is submitted to two further extractions with 300 cc. portions of 50 % alcohol. The three filtered alcoholic extracts are combined and reserved.

The filtered residue, thus freed from pectin and much of its lignin, is allowed to stand at room temperature with 300 cc. of 4 % NaOH solution for not less than 6 hours. The extract is then filtered at the pump through a circle of calico previously boiled with 4 % NaOH. The wood residue is repeatedly further extracted with 150 cc. portions of NaOH solution until the filtered extract no longer gives a precipitate on neutralising with glacial acetic acid and adding an equal volume of acetone.

The combined filtrates are rendered acid by addition of glacial acetic acid, and a volume of acetone equal to that of the acid liquid is added. The precipitate is allowed to settle, the supernatant liquor syphoned off and the hemicellulose treated with graded strengths of alcohol, drying being continued *in vacuo* after filtering at the pump. The weight of the product is ascertained, its moisture content determined on a small portion by oven drying, and the yield further corrected for ash content. The yield of dry, ash-free hemicellulose is calculated as a percentage of the dry wood, being returned as "free hemicellulose," *i.e.* hemicellulose soluble in 4 % sodium hydroxide solution in the cold.

By this method, the losses of material resulting during the estimation can be reduced to negligible proportions. It is suggested that the free hemicellulose content of woody materials can be more accurately determined in this way than by other methods.

The wood residue, after removal of free hemicellulose, is treated with 300 cc. of boiling 4 % NaOH solution for 1 hour, the extract being filtered clear on calico as before. The residue is then repeatedly extracted with 150 cc. portions of boiling 4 % NaOH solution, till the filtered extract on cooling no longer gives any appreciable amount of precipitate on neutralising with glacial acetic acid and adding an equal volume of acetone.

The combined filtered extracts are neutralised and acetone added as above. The precipitate obtained is taken up to dryness in the usual way and is designated "combined hemicellulose."

It should be understood that the terms "free" and "combined" hemicellulose are employed relatively, rather than literally, to emphasise the more drastic treatment necessary to remove the latter.

The alkaline alcoholic filtrates reserved from above are rendered acid by the addition of glacial acetic acid, and 95 % alcohol may be further added to assist precipitation. The precipitate which separates out after prolonged standing is re-dissolved in 1 % NaOH, the solution is acidified, and alcohol

added to give a concentration of 75 %. The precipitate is allowed to settle, the liquid syphoned off and the product taken to dryness in the usual way.

This product, which appears to possess many properties in common with the hemicelluloses, is here described as "lignosaccharide," indicating its extraction with alcoholic sodium hydroxide, a solvent which readily extracts a portion of the lignin of woody tissues.

It is inconvenient to deduce the lignosaccharide content of the tissue from the yield obtained in this preparation, as the possibility of loss of product appears to be great. Consequently, the furfuraldehyde yield of the substance is determined, and its amount present in the wood calculated by the method shown below.

The results to this point are set out in Table II.

Table II.

Calculated on dry wood:	free hemicellulose	7·68 %
	combined hemicellulose	6·43
Furfuraldehyde yield from:	free hemicellulose	47·35 %
	combined hemicellulose	51·44
	lignosaccharide	38·10

The second stage of the estimation involves the treatment of 3–5 g. of the material with 200 cc. of 0·5 % ammonium oxalate solution at 85° for 24 hours. The solution is filtered, the residue washed with further ammonium oxalate solution, and the filtrate and washings are made up to 250 cc. The tissue residue is reserved. 100 cc. of the extract are treated for the estimation of total pectin by the method of Nanji and Norman [1928]. A second 100 cc. portion of the extract is evaporated gently to 50 cc., and 25 cc. of concentrated hydrochloric acid are added. The whole is washed into a distilling flask with 12 % HCl, and furfuraldehyde distillation carried out in the usual way. The furfuraldehyde arising is estimated and calculated to a percentage of the original dry wood.

This furfuraldehyde arises from pectin and from non-pectic substances simultaneously removed. The amount of pectin present being known, the non-pectic furfuraldehyde may be calculated by difference from the fact that 100 parts of calcium pectate yield 19·5 parts of furfuraldehyde.

The reserved tissue residue is extracted twice with 100 cc. portions of boiling 50 % alcohol containing 1 % of NaOH, the periods of extraction each being 1½ hours. The alcoholic filtrates are combined, and the tissue is finally washed with hot 50 % alcohol, the washings being added to the combined filtrates. To the extract is added glacial acetic acid so that the mixture remains just alkaline, the alcohol is evaporated off, and the total volume adjusted to 50 cc. 25 cc. of concentrated HCl are added, and the mixture is washed into a distilling flask, and furfuraldehyde again estimated and calculated to the dry wood.

Knowing now the amount of furfuraldehyde obtainable from the alcoholic

sodium hydroxide extract, and also the furfuraldehyde yield from the lignosaccharide, the percentage of this constituent in the tissue can be calculated. Thus, in the case of box-wood, the figures were 0·70 % of furfuraldehyde from the extract, and 38·10 % from the lignosaccharide, whence this wood contains 0·70/0·381 or 1·83 % of lignosaccharide.

If now a total furfuraldehyde determination is carried out on the wood itself, a complete furfuraldehyde balance sheet can be drawn up as shown in Table III.

Table III.

	% of dry wood
Furfuraldehyde from substances soluble in:	
(i) 0·5 % ammonium oxalate (*a*) pectin ...	0·08
(*b*) non-pectin	0·18
(ii) Alcoholic sodium hydroxide	0·70
Furfuraldehyde from:	
(iii) Free hemicellulose	3·64
(iv) Combined hemicellulose	3·31
(v) Other sources (by difference)	3·95
Total furfuraldehyde found directly	11·86

It was shown that the material remaining after removal of hemicelluloses from box-wood still retained a considerable amount of xylan.

Some 2 g. of the wood residue was treated with 72 % sulphuric acid according to the method of Monier-Williams [1921]. The dried products of hydrolysis were extracted with absolute alcohol. The bulk of the sugar produced was dissolved in this way and proved to be a mixture of glucose and xylose. Thus it yielded cadmium xylonobromide and a mixture of glucosazone, melting at 205°, and xylosazone melting at 155°. Other sugars could not be detected.

Consequently, the furfuraldehyde described as from "other sources" is converted to xylan, using the factor 1·55, the figure appearing as "other pentosans" in Table IV corresponding to this result.

Cellulose. This constituent was determined by chlorination, after a preliminary treatment of the tissue with 100 cc. of 1 % NaOH solution for 20 minutes. Chlorination was carried out in a Gooch crucible for periods of 20 and 15 minutes, followed by two further treatments of 10 minutes each. The resulting crude cellulose in every case gave large yields of furfuraldehyde on distillation with 12 % HCl. The yield of crude cellulose was therefore corrected, as suggested by Norman [1929], on the assumption that this furfuraldehyde is derived from xylan associated with the cellulose, the factor 1·55 being used for conversion of furfuraldehyde yield to xylan.

A further correction might conceivably be made assuming that the difference between furfuraldehyde yield from the crude cellulose, calculated back to the dry wood, and the furfuraldehyde from "other sources" of Table III represents hemicellulose not removed by chlorination. However, it is not unlikely that different portions of the hemicellulose complex might show

different degrees of resistance to removal by chlorination, and further correction on these lines was not attempted.

Lignin. It was recognised by Mehta [1925] that lignin estimation by alkaline treatment gave results which were very much lower than those obtained by the use of strong mineral acids.

Undoubtedly, some change is induced in lignin by acid treatment. Thus, lignin obtained by using 72 % sulphuric acid may still contain small amounts of carbohydrate material, while evidence has been obtained by Mehta that sulphonation may also take place. On the other hand, methoxyl and acetyl groups may be lost during the treatment, and on the whole it is not unlikely that the various errors tend to balance one another [*cf.* Euler, 1923].

In the present work, two methods of lignin estimation are used. The first is Mehta's method for free and combined lignin, these being returned separately. The second, carried out on wood previously extracted with alcohol, employs the 72 % H_2SO_4-HCl mixture of Schwalbe [1925] in the manner described by Norman [1929]. The probable relative value of the two methods is further discussed below.

Other constituents. Ash content is determined in the usual way. The loss in weight of the wood when exhaustively extracted with alcohol and ether successively is also found. If the acid method of lignin estimation is used, the value for total "alcohol-ether-soluble" is returned directly. If free and combined lignin are determined by alkaline treatment and returned separately, the value for free lignin must be subtracted from total alcohol-ether-soluble, and the difference returned as "free fat, pigment, *etc.*" The residue after alcohol-ether extraction is used for the determination of protein, the factor 6·25 being employed.

Finally, hot water-soluble substances are determined as described by Schorger [1926, p. 506]. If overlapping of results is to be avoided, "hot water-soluble" may not be returned directly. Thus, hot water dissolves some of the ash constituents, possibly protein, and some of the material soluble in alcohol and ether. Accordingly, these determinations are repeated on wood extracted with hot water, and the value for "hot water-soluble" is corrected. In some cases, the pectin soluble in hot water might also have to be taken into account, but it was not thought necessary to attempt this further correction in the case of woods.

Results of analysis.

The results obtained by the above methods of analysis are set out in Table IV. Two columns are given, allowing the values obtained by the two methods of lignin estimation to appear in separate columns, together with the correction where necessary of "alcohol-ether-soluble" to "free fat, pigment, *etc.*"

The summation of 87·78 % obtained when lignin is estimated by sodium hydroxide treatment is most unsatisfactory, since it is inconceivable that any constituent present in appreciable amount has escaped determination. Using

Table IV.

	% of dry wood	
Ash	1·22	1·22
Alcohol-ether-soluble ...	—	3·91
Free fat, pigment, *etc.* ...	2·05	—
Protein	2·81	2·81
Hot water-soluble (corrected)	0·71	0·71
Free lignin (NaOH)	1·86	—
Combined lignin (NaOH) ...	15·23	—
Lignin (H_2SO_4-HCl)	—	26·45
Pure cellulose	41·43	41·43
Calcium pectate number ...	0·41	0·41
Free hemicellulose	7·68	7·68
Combined hemicellulose ...	6·43	6·43
Lignosaccharide	1·83	1·83
Other pentosans (as xylan)...	6·12	6·12
Total estimated	87·78	99·00

the acid method, on the other hand, the summation of 99·00 % represents a very satisfactory figure.

On this evidence, it is reasonable to conclude that figures for lignin content obtained by the alkali method cannot be accepted as reliable, and it may be mentioned that figures further supporting this view have been obtained with other woods.

B. Nature of the furfuraldehyde-yielding constituents of box-wood.

There remain to be discussed the results of a qualitative study of the furfuraldehyde-yielding constituents of the wood.

Water-soluble constituents. 100 g. of the sawdust were boiled for several hours with 1 litre of water. The filtered extract was cooled and concentrated to a bulk of 50 cc. 200 cc. of absolute alcohol were added, and after settling the supernatant liquor was poured off and reserved. The precipitate was heated with absolute alcohol to denature any protein present, and the residue was extracted with hot water, and the solution filtered. Four volumes of absolute alcohol were added to the filtrate, this treatment bringing down a very small amount of precipitate.

The product was readily soluble in hot water, the solution being non-reducing. It gave a blue-green colour with iodine solution, suggesting the presence of a little starch or amylo-hemicellulose [*cf.* Ling and Nanji, 1925]. On treating a little of the solution with Bial's orcinol reagent [see Browne, 1912, p. 382] the reaction for pentoses gave a negative result, but on boiling the solution a green colour developed, suggesting the presence of uronic anhydride. Possibly the substance responsible for this reaction is analogous to the ϵ-galactan of Trimble [1898] and of Schorger and Smith [1916], who isolated from western larch a substance yielding furfuraldehyde, but containing no pentosan. Their product was probably uronogalactan. The amount arising from box-wood was insufficient for examination.

The mother-liquor reserved from above was evaporated just to dryness *in vacuo* and the residue taken up in water. The solution, which had strong reducing properties, gave no coloration with iodine and was free from furfuraldehyde-yielding substances. Free simple sugars were evidently absent, since a crystalline osazone could not be obtained; the product instead was gelatinous, and its properties resembled those of phenylhydrazine derivatives prepared from the dextrinous products of starch conversions.

Lignosaccharide. The method of preparation of this substance has already been described, 100 g. of box-wood yielding 1·5 g. of lignosaccharide. The product was a fine white powder, somewhat soluble in cold water, completely in hot water to an opalescent, non-reducing solution. Lignosaccharide dissolves to a clear solution in 4 % NaOH solution and is not precipitated by Fehling's solution. It is precipitated, however, from the mixture on addition of acetone, thus behaving analogously to hemicelluloses B2 and C2 isolated from other sources.

Box-wood lignosaccharide gave 38·10 % of furfuraldehyde, and 6·65 % of carbon dioxide, the latter corresponding to a uronic anhydride content of 26·60 %.

This product showed a marked degree of resistance to hydrolysis. Thus, boiling for 2 hours with 5 % sulphuric acid produced a liquor in which free simple sugars could scarcely be detected.

Boiling for 7½ hours proved more successful, sugar being produced equivalent in amount to the hydrolysis of rather less than one-half of the lignosaccharide.

Two-thirds of this sugar was dissolved by absolute alcohol and proved to be chiefly xylose.

The sugar soluble in 80 % alcohol was treated with nitric acid for the preparation of mucic acid, but with negative results, so that the only constituents of the lignosaccharide which have been identified are uronic anhydride and anhydro-xylose.

Free hemicellulose. The wood residue remaining after removal of water-soluble substances, pectin, lignin, and lignosaccharide was treated with 4 % NaOH solution at room temperature, and the filtered extract was neutralised with glacial acetic acid and submitted to acetone fractionation. Only one fraction was obtained, corresponding to crude hemicellulose B, the yield from 100 g. of sawdust being 6·2 g. This was purified by copper treatment, the whole being precipitated from alkaline solution by Fehling's solution. Thus, the only free hemicellulose obtainable from box-wood sawdust is hemicellulose B1.

The purified product is a white, amorphous powder, easily soluble in hot water, the solution giving a faint opalescence on cooling. The solution is non-reducing and gives a faint greenish tinge with iodine. The hemicellulose is precipitated from aqueous solution by basic, but not by neutral, lead acetate. Three-quarter saturation with ammonium sulphate also gives a precipitate.

The hemicellulose is easily soluble in cold dilute alkali and in hot dilute mineral acids, the latter causing hydrolysis. The solution in 4 % NaOH solution gives for $c = 3{\cdot}00$ $[\alpha]_D^{20^\circ} - 62{\cdot}3^\circ$.

An aqueous solution of this hemicellulose is hydrolysed by the action of taka-diastase. After 24 hours, a precipitate is no longer given on rendering the solution alkaline and adding Fehling's solution, but the subsequent addition of acetone gives a thick precipitate. Iodine absorption corresponding to hydrolysis of 11 % of the hemicellulose takes place at this stage. At the end of 7 days, 40 % of the hemicellulose is apparently hydrolysed, and an equilibrium is reached at a little in excess of this value after 10 days. At the end of this time, the amount of precipitate given by the solution, either with acetone or a mixture of Fehling's solution and acetone, is extremely small. Free simple sugars cannot be detected in the final hydrolysis liquor.

The following analytical figures were obtained with hemicellulose B1, being calculated to a dry, ash-free basis:

Yield of furfuraldehyde	55·14 %
,, carbon dioxide	4·25 %
Hence, uronic anhydride	17·00 %

Since, as is shown below, the only sugar which can be detected amongst the products of hydrolysis of this substance is xylose, these figures may be interpreted as follows:

Furfuraldehyde from uronic anhydride	2·83 %
Hence, furfuraldehyde from xylan	52·31 %

The composition of the hemicellulose may now be calculated:

Uronic anhydride	17·00 %
Xylan	81·08 %
Total accounted for	98·08 %

This composition corresponds approximately to a molecule

$$\{(C_5H_8O_4)_6 \cdot (C_6H_8O_6)\}_n$$

which would yield 55·80 % of furfuraldehyde, and 4·55 % of carbon dioxide.

1·31 g. of the hemicellulose was hydrolysed with 75 cc. of 1 % sulphuric acid for 3½–4 hours. The sulphuric acid was neutralised with the calculated quantity of 0·25N barium hydroxide solution, and the filtered liquid, free from barium sulphate, titrated with 0·25N barium hydroxide till it remained just acid to phenolphthalein. 4·7 cc. were required to effect this, equivalent to $0{\cdot}176 \times 0{\cdot}25 \times 4{\cdot}7 = 0{\cdot}207$ g. of uronic anhydride, *i.e.* 15·7 % calculated on the hemicellulose. But the hemicellulose contains 17·0 % of uronic anhydride; hence, a practically quantitative liberation of the free carboxyl groups had been achieved. No insoluble barium salt was thrown down during this neutralisation of carboxyl groups.

The solution was taken just to dryness *in vacuo* and the residue extracted

several times with absolute alcohol. Xylose was isolated from the alcoholic extracts. The dry product weighed 0·55 g., corresponding to a yield of some 45 % of the theoretical. The xylose obtained gave $[\alpha]_D^{20°} + 18·6°$, and the usual derivatives were prepared.

It has been shown in the case of the hemicelluloses of maize cobs [Preece, 1930] that 90 % of the theoretical amount of xylose can be obtained by the action of 1 % sulphuric acid. Hence, it appears fairly certain that acid of this strength does not cause a complete hydrolysis of box-wood hemicellulose; indeed, it seems to liberate only about one-half of the total possible amount of xylose.

Accordingly, the formula given above may be amended to

$$[(C_5H_8O_4)_3 \cdot \{(C_5H_8O_4)_3 \cdot (C_6H_8O_6)\}]_n$$

where one-half of the anhydro-xylose exists in a form of combination differing from that of the rest.

To test these suggestions further, a known weight of hemicellulose was treated with 1 % sulphuric acid, portions of the hydrolysis liquor being removed every half-hour, and the sugar present estimated iodimetrically by the method of Baker and Hulton [1920]. The results were calculated as apparent xylan and further expressed as a percentage of the hemicellulose hydrolysed. In a typical experiment, 1·473 g. of dry, ash-free hemicellulose was heated with 100 cc. of 1 % sulphuric acid.

Table V A.

Time from commencement hrs.	Apparent xylose produced g.	Equivalent weight xylan g.	Xylan as % of hemicellulose
½	0·515	0·453	30·7
1	0·883	0·777	52·7
1½	1·024	0·901	61·2
2	1·065	0·938	63·7
2½	1·059	0·932	63·3

It is apparent from this table that an equilibrium is reached when there is a production of apparent xylose equivalent to the hydrolysis of some 64 % of the hemicellulose. However, the percentage of free xylose liberated in the first experiment described corresponds to a hydrolysis of xylan calculated on the hemicellulose of only 41 %. The difference between 64 % and 41 %, therefore, must be attributed to a liberation of free aldehydic groups, capable of reacting with alkaline iodine, but not present in the form of free xylose.

In continuation of the above hydrolysis, the volume of hydrolysis liquor

Table V B.

Time from commencement hrs.	Apparent xylose produced g.	Equivalent weight xylan g.	Xylan as % of hemicellulose
3	0·799	0·703	79·5
3½	0·827	0·728	82·4
4	0·840	0·740	83·7
4½	0·875	0·771	87·2

remaining (60 cc.) was treated after $2\frac{3}{4}$ hours with 40 cc. of 10 % sulphuric acid, the resultant concentration of acid thus being 4·6 %, and 100 cc. of hydrolysis liquor now being equivalent to 0·883 g. of hemicellulose (see Table V B).

Thus, the increased concentration of acid caused an increase in the amount of hydrolysis, the rate, however, diminishing after the first few minutes.

Next the course of hydrolysis was followed, using 4·6 % sulphuric acid from the commencement. The equivalent of 0·7237 g. of dry, ash-free hemicellulose was treated with 50 cc. of 4·6 % sulphuric acid.

Table VI.

Time from commencement hrs.	Apparent xylose produced g.	Equivalent weight xylan g.	Xylan as % of hemicellulose
$\frac{1}{2}$	0·473	0·416	57·5
1	0·652	0·574	79·3
$1\frac{1}{2}$	0·652	0·574	79·3
2	0·641	0·564	77·9
$2\frac{1}{2}$	0·613	0·540	74·6
3	0·673	0·592	81·8
$3\frac{1}{2}$	0·684	0·602	83·1
4	0·652	0·574	79·3

Here again the hydrolysis at first proceeds rapidly, but after 1 hour further action is slow, and eventually shows a falling off. This last effect is probably to be attributed to a destruction of uronic acid. Thus, O'Dwyer [1926] found that uronic acids tend to decompose during hydrolysis. This effect masks for a time the liberation of sugar, which, however, eventually re-asserts itself. It should be noted that the point at which the hydrolysis slackens—79·3 %—is almost exactly the same as the point of retardation found in the previous experiment—approximately 79·5 %.

Hydrolysis of the hemicellulose with an intermediate strength of sulphuric acid, namely 2·8 %, gave results agreeing closely with those obtained with 1 % acid, equilibrium being reached at an apparent hydrolysis of about 66 % of the hemicellulose.

Hydrolysis with either 1 % or 2·8 % acid, therefore, causes the rapid apparent hydrolysis of 64–66 % of the hemicellulose, subsequent action being absent or very slow. 4·6 % acid causes the rapid apparent hydrolysis of approximately 80 % of the hemicellulose, the action proceeding slowly beyond this point, when its effect may be at least partially masked by a destruction of uronic acid.

The results obtained may be best interpreted as follows. Suppose the structure of the hemicellulose is represented by the formula

$$\underset{\text{I}}{\{(C_5H_8O_4)_3} \cdot \underset{\text{II}}{(C_5H_8O_4)_2} \cdot \underset{\text{III}}{(C_5H_8O_4 \cdot C_6H_8O_6)\}_n}.$$

It is to be assumed that the following changes take place. I is rapidly hydrolysed by 1 % H_2SO_4 to free xylose. II is converted to a disaccharide of

the form $C_5H_9O_4.O.C_5H_9O_4$ or, since it contains a free aldehydic group, $CHO.C_4H_8O_3.O.C_5H_9O_4$. III is converted to a similar disaccharide derivative, containing one free aldehydic group, and one free carboxyl group. This would account for the liberation of the total acidity without formation of free uronic acid. It appears likely that the uronic acid concerned is glycuronic acid, and indeed free glycuronic acid could not be detected amongst the products of hydrolysis of the hemicellulose by 1 % acid.

The suggestion that a disaccharide derivative of the type of a xylosido-uronic acid is formed may be compared with the observation of Butler and Cretcher [1929] that hydrolysis of arabic acid, obtained from gum arabic, with 2 % sulphuric acid yields *d*-galactosido-*d*-glycuronic acid.

Hydrolysis of the hemicellulose in this way would result in the liberation of three molecules of xylose, one free carboxyl group, and two free additional .CHO groups.

4·6 % acid causes a further liberation of .CHO groups. Suppose the portion III of the molecule is, along with I, readily hydrolysed to its components by this stronger acid, while II is still only slowly attacked. Then a total of 4 molecules of free xylose, one of uronic acid (now susceptible to the destructive action of the mineral acid), and one additonal free .CHO group would be liberated.

Assuming that 90 % of the free xylose produced is isolated as such, the theoretical yields on the foregoing assumptions and the yields actually obtained are given in Table VII.

Table VII.

	1 % H_2SO_4		4·6 % H_2SO_4	
	Free xylose from 1 g. of hemicellulose	Total apparent hydrolysis*	Free xylose from 1 g. of hemicellulose	Total apparent hydrolysis*
Theory	0·42 g.	68 %	0·56 g.	82 %
Actual	0·42	65	0·52	80

* Calculated as a percentage of xylan on the hemicellulose.

Thus, there appears to be good evidence for assuming that the hemicellulose is a polymerised substance of the formula given above.

Combined hemicelluloses. The tissue residue remaining after removal of free hemicelluloses was extracted several times with boiling 4 % NaOH solution, the extracts obtained being filtered and combined. Neutralisation of the cooled extracts with glacial acetic acid caused a precipitation of combined hemicellulose fraction A. This was centrifuged off, and half a volume of acetone was added to the filtered centrifugate. Fraction B was thus precipitated. The filtrate from this gave a further small amount of precipitate on adding more acetone, but the yield of fraction C arising in this way was too small for examination.

Heated with water, fraction A gives a silky suspension, which is non-reducing and gives a greenish tinge with iodine. This hemicellulose becomes

soluble in cold 4 % NaOH, and the bulk of it is precipitated from alkaline solution on addition of Fehling's solution. A separation on these lines into its components A1 and A2 was not attempted, owing to the very small amount of A2 which would thus have been obtained.

Fraction B is soluble in hot water; the solution gives a pale green colour with iodine and is non-reducing. This fraction also dissolves in cold 4 % NaOH solution and is partially re-precipitated on addition of Fehling's solution. Again, a separation was not made.

The usual analytical data were obtained, as shown in the following table.

Table VIII.

Hemicellulose fraction	A	B
Furfuraldehyde yield	36·57 %	48·29 %
Carbon dioxide yield	3·22	4·33
Uronic anhydride content	12·88	17·32

In each case hydrolysis was carried out by boiling for 2 hours with 5 % sulphuric acid. After removal of sulphuric acid, the hydrolysis liquid was evaporated just to dryness *in vacuo*, and the sugar extracted successively with absolute and 80 % alcohol. Several extractions were made with alcohol of each strength. The filtered extracts were worked up for the preparation of dry sugar fractions.

Some 65 % of the sugar obtained from combined hemicellulose A was dissolved by absolute alcohol. This product had $[\alpha]_D^{20^\circ} + 17^\circ$ and represented an impure sample of xylose. The sugar dissolving in 80 % alcohol gave $[\alpha]_D^{20^\circ} + 26^\circ$, but sugars other than xylose could not be detected even in this fraction.

In the case of combined hemicellulose B also the bulk of the sugar produced on hydrolysis was dissolved by absolute alcohol. The sugar of this fraction was again impure xylose.

80 % alcohol dissolved sugar having $[\alpha]_D^{20^\circ} + 49^\circ$. The osazone prepared from the sugar of this fraction was a mixture of indefinite character and low melting-point, but certainly contained much xylosazone. An attempt to prepare cadmium xylonobromide was successful. From the figure quoted and the fact that xylose is present to an appreciable extent in the sugar of this fraction, it would seem that the second sugar of this fraction is one with a high specific rotation. However, mucic acid tests gave negative results, while the absence of arabinose from the hydrolysis products is indicated by the low specific rotation of the sugar of the absolute alcohol-soluble fraction, namely $[\alpha]_D^{20^\circ} + 20^\circ$. Thus the nature of the second sugar must remain at present undetermined.

The tissue residue. The wood residue remaining after removal of combined hemicelluloses was examined. Attempted estimations of mannan and galactan were carried out by the methods described by Schorger [1926, pp. 537, 538], but both these substances were found to be absent. As has been shown above,

the pentosan constituent of the residue is xylan. It was also found that boiling dilute acid—5 % sulphuric acid—had but little hydrolytic effect on the wood residue.

Discussion.

Since the amount of pectin present in the wood is 0·41 %, the proportion of anhydro-arabinose is less than 0·1 %. Anhydro-xylose, on the other hand, amounts to approximately 17 % of the wood, while the total uronic anhydride content is of the order of 3 %. Unidentified anhydro-hexose or anhydro-methylpentose constituents of the hemicelluloses and lignosaccharide account for some 2 % of the wood.

It is apparent from the work described that there is little difference in nature between the combined hemicelluloses isolated from box-wood and the free hemicelluloses from box-wood and from the other sources dealt with previously [Norris and Preece, 1930; Preece, 1930, 1931]. The lignosaccharide, though more resistant to hydrolysis, is also of a similar nature. These constituents, consequently, can be grouped together as "structural hemicelluloses," the name "hemicellulose" being retained in preference to that of "polyuronide" suggested by Candlin and Schryver [1928].

It is desirable to distinguish between the hemicelluloses and the xylan of the wood residue. This xylan resists extraction by the relatively drastic action of boiling 4 % NaOH solution and is also resistant to the hydrolytic action of boiling dilute mineral acids. In short, its properties approach more nearly those of cellulose than those of the hemicelluloses. Accordingly, it is proposed to make such a distinction by the re-introduction of the term "para-xylan" for this resistant form of xylan. Thus, the final wood residue may be said to consist of cellulose and para-xylan, together with some unextracted lignin.

Although it has been found possible to assign a comparatively simple formula to the free hemicellulose of box-wood, it is not claimed that the fractions described in this and previous communications represent individual hemicelluloses [see also Preece, 1930]. It has been shown by O'Dwyer [1928] that the complexity of a hemicellulose seems to vary with the age of the wood from which it is obtained. If change in complexity is regularly taking place, this must lead to the presence of a large number of true hemicellulose units of different composition. Since the present method of separation of free hemicelluloses cannot give more than six fractions, it would seem that these fractions must, generally speaking, be mixtures, representing aggregates of molecules of similar physical properties.

A similar argument might, it may be supposed, be applied to lignosaccharide and combined hemicelluloses. An inter-relationship between these three subdivisions of the structural hemicelluloses might conceivably, if shown to exist, lead to a revision of the above view.

SUMMARY.

1. A method of analysis of woody tissues giving a very satisfactory summation is described, various types of furfuraldehyde-yielding substance being estimated separately.

2. The alkali method for lignin estimation gives results which cannot be accepted.

3. Free pentoses are absent from box-wood, but hot water extracts some non-pentosan furfuraldehyde-yielding substance.

4. Lignosaccharide and free and combined hemicelluloses have been isolated from box-wood, and their nature examined. Xylose and uronic acid arise on hydrolysis of each of these substances.

5. The composition of the free hemicellulose is completely accounted for as urono-xylan of relatively simple constitution. The hydrolysis of this substance has been closely studied, and different parts of the molecule shown to possess different degrees of resistance to hydrolysis by dilute sulphuric acid.

6. The wood residue remaining after extraction of the above constituents consists chiefly of cellulose, para-xylan, and some unextracted lignin.

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CXLIV. THE OCCURRENCE OF PYRUVIC ACID AND METHYLGLYOXAL IN MUSCLE METABOLISM.

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That the metabolic changes which occur in muscle are of profound complexity is evident. This work was undertaken with the object of studying some of the intermediary mechanisms of muscle carbohydrate metabolism by means of the use of reagents which would remove from the system some product which is only transitorily formed normally. The method is essentially the same as that employed by Neuberg in his study of the mechanisms involved in alcoholic fermentation. The fixative of which we have availed ourselves, namely sodium sulphite, is the same as that used by Neuberg in what constitutes his second form of fermentation, in which acetaldehyde is "fixed" as the bisulphite compound. It is well known that substances of an aldehydic or ketonic nature possess the property of forming addition compounds with sodium bisulphite. The conditions that determine the stability or otherwise of these compounds to further metabolic change have not, however, been worked out. In the conditions under which we have worked, pyruvic acid and methylglyoxal will form with bisulphite compounds that are relatively stable in this regard.

Neuberg and Reinfurth [1920] have shown that in the case of yeast the pyruvate-bisulphite complex is decarboxylated to acetaldehyde. Cook [1930] has confirmed this observation for yeast but has noted that *B. coli communis*, for example, is unable to bring about this decarboxylation. It was also noted that if oxidations of various substrates were carried out in the presence of sulphite, the sulphite served as a very effective fixative for any pyruvic acid that was formed. The method of sulphite fixation also furnishes evidence as to the occurrence of other aldehydic or ketonic compounds during the course of metabolism.

It may be, and often is, urged that the fixation method is liable to give a false impression of the chemical events taking place in that it tends to endow subsidiary reactions with a spurious prominence. It will be shown, however, that as regards the carbohydrate metabolism of muscle it is possible, if sufficient care be taken, to identify pyruvic acid in normal muscle in the absence of any fixatives, and in amounts commensurate with those found when a fixative is

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present. It is only to be expected, nevertheless, that under normal conditions the pyruvic acid, if of importance in metabolism, would quickly disappear. As will be shown later, this disappearance is indeed extremely rapid in the normal muscle.

Although there is no doubt that the presence of sulphite does affect the normal balance of reactions, we are of the opinion that this method of fixation affords useful indications concerning some of the chemical events associated with the metabolism of muscle.

The oxidation of lactate by muscle tissue.

It was postulated by Meyerhof, and the view commands general acceptance, that part of the lactic acid in muscle is oxidised in order to provide energy for the resynthesis of the remainder to carbohydrate. This, taken in conjunction with the fact that lactic acid is dehydrogenated to pyruvic acid by muscle tissue in the presence of methylene blue [Hahn, Fischbach and Haarman, 1928] possibly indicates that at least part of the lactic acid would, under aerobic conditions, with atmospheric oxygen as the hydrogen acceptor, be oxidised *via* pyruvic acid. In the instance of the oxidation of lactate by *B. coli*, pyruvate may be fixed by the use of sulphite, the oxidation (measured as oxygen uptake) corresponding to 72 % transformation of lactate to pyruvate [Cook, 1930]. It was hoped that by the employment of similar methods with muscle, any pyruvic acid which was formed would be made evident by the presence of the sulphite.

As a preliminary it was desirable to investigate the effect of the addition of lactate on the normal respiration of muscle. The respiration was measured as the oxygen uptake in the Barcroft differential manometer. The left-hand pot contained 1·5 cc. frog Ringer solution, 1·0 cc. phosphate buffer (p_H 7·2) and 0·5 cc. water. The right-hand pot contained 1·5 cc. Ringer solution, 1·0 cc. buffer and 0·5 cc. water or $M/10$ lactate solution, together with a weighed amount of frog muscle. Each of the small central pots contained a roll of filter-paper soaked in 6 % potassium hydroxide to absorb CO_2.

It is evident that the metabolism of the muscle is affected by the physiological conditions of the tissue, *i.e.* is altered by circumstances such as chopping, mincing or washing. Some of the results obtained are shown in Table I.

It will be seen that there appears to be a slightly greater oxygen uptake with intact muscle on the addition of a solution of sodium lactate (Exp. 1). On the other hand the addition of lactate to unwashed chopped muscle has little effect on the oxygen uptake (Exp. 3). There is a slightly increased uptake upon the addition of lactate to washed chopped muscle (Exp. 5), but it is doubted if this is significant. The addition of sulphite causes a marked decrease in the oxygen uptake both with intact and chopped muscle (Exps. 2 and 4).

The initial velocities of oxidation of the intact gastrocnemii were the same in the two instances, but after 3 hours a small but appreciable difference was noted in the rate of oxygen uptake of the control as compared with the muscle

Table I.

				Controls Ringer + phosphate p_H 7·2				Ringer + phosphate + $M/60$ Na_2SO_3 p_H 7·2			
						+ $M/60$ lactate				+ $M/60$ lactate	
Remarks	No. of exp.	Temp. (°)	Duration of exp. (hrs.)	Wt. of muscle (g.)	O_2 (mm.³)	Wt. of muscle (g.)	O_2 (mm.³)	Wt. of muscle (g.)	O_2 (mm.³)	Wt. of muscle (g.)	O_2 (mm.³)
Intact (gastrocnemii)	1	24	4·75	0·79	208·5	0·76	230·8	—	—	—	—
	2	25	4	0·49	125	—	—	0·56	31·5	0·53	60·9
Chopped unwashed	3	23·5	2·5	0·5	336	0·5	340	—	—	—	—
	4	19·5	3·5	—	—	0·5	238	—	—	0·5	74·1
Chopped washed	5	23·5	4	0·5	61·6	0·5	73·2	—	—	—	—

to which lactate had been added. It is evident therefore that some oxidation of the added lactate has taken place.

It is realised that in the oxidation of lactate by muscle no increase in velocity would be expected in either intact or unwashed chopped muscles, as the enzymes dealing with the oxidation of lactate would be saturated with this substance; hence no difference in velocity could be expected as long as this amount remains in excess. None of our experiments was of more than 6 hours' duration and in no case was the oxidation in the presence of lactate very much greater than that in its absence. It is clear that under our conditions at least, the oxidation of lactate by muscle is a relatively slow process.

The muscles in Exps. 2 and 4 were removed from the pots after 4 hours, rapidly ground with sand and filtered through muslin. Tests for pyruvate were then carried out by the method of Simon and Piaux [1924]. (A description of this is given later.) Slight positive reactions were given by the intact muscle and unwashed chopped muscle in the presence of sulphite, the controls in which no sulphite was present being definitely negative.

Some estimations were carried out on the disappearance of lactic acid in the systems both with and without the addition of sulphite. The results obtained were however rather indefinite, but it appears that the oxidation of lactate constitutes only a small percentage of the total respiration. Under the conditions described the Friedemann, Cotonio and Shaffer [1927] method for the determination of lactic acid is rather unsatisfactory. It is hoped to give further details of the relation of pyruvic acid formation to lactic acid oxidation in a later paper.

Methods for the detection and estimation of compounds formed.

As an indication of the formation of pyruvic acid we have generally employed the test of Simon and Piaux [1924]. Considerable caution must, of course, always be exercised in using a colour reaction as the sole evidence of formation of any particular compound. As will appear later we have not relied exclusively upon colour tests, and we have invariably found that the indications afforded by other methods have run closely parallel with those obtained

by the Simon and Piaux reaction. As a qualitative indication of the presence of pyruvic acid this test is carried out as follows. About 3 cc. of the material to be examined are placed in a test-tube and ammonium sulphate is added in such quantity that a layer rests at the bottom of the tube. Crystals of sodium nitroprusside are sprinkled on this layer and an equal volume of ammonia (sp. gr. 0·880) is then added without shaking. In the presence of filtrates prepared from muscle a pink colour at first makes its appearance. This reaction is due to the .SH compounds present, but rapidly fades and does not interfere with the pyruvic acid reaction. This latter reaction appears about half an hour after the commencement of the test and consists in a blue or greenish-blue colour, which develops in the neighbourhood of the nitroprusside crystals, and if the concentration of pyruvate be sufficiently great spreads throughout the solution. The colour increases in intensity for about 2 hours, after which it begins to fade, and with higher concentrations of pyruvate a red colour makes its appearance after the lapse of about 12 hours. Although nitroprusside and alkali hydroxides give various colour reactions with numerous aldehydes and ketones and certain unsaturated sulphur derivatives, we are not aware of any substance of likely physiological importance, other than pyruvic acid, which gives with nitroprusside and ammonia the characteristic blue-green colour; and we are of the opinion that this test is a reliable criterion for the presence of pyruvic acid. We have tested the reaction with a solution of methylglyoxal but have not obtained a positive result. With nitroprusside and alkali hydroxide methylglyoxal gives a red colour which is however insufficiently specific to constitute a useful test.

Needless to say, any satisfactory proof of the formation of a compound must involve either the isolation of the actual compound or the isolation of some convenient derivative. The first alternative is in the present case obviously rather difficult to undertake. For the purposes of identification we have used *p*-nitrophenylhydrazine and 2 : 4-dinitrophenylhydrazine. The method used in the earlier experiments was to precipitate the proteins with trichloroacetic acid and then to add drop by drop a freshly prepared saturated solution of *p*-nitrophenylhydrazine in glacial acetic acid. This has the disadvantages that in the first place considerable concentration of the material under test is necessary, and that in the second place precipitation of the reagent itself is apt to occur when the acid solution is added even to water alone. Hence the precipitates were frequently contaminated with considerable amounts of unchanged reagent, which tended to interfere with the subsequent colour reactions with alcoholic potassium hydroxide. Nevertheless, since we at first used this substance, we have prepared the derivatives of pyruvic acid and methylglyoxal from these compounds themselves, and have for convenience incorporated some of their properties in Table II. For the separation of these derivatives we have followed the method described fully by Dakin and Dudley [1913].

It was found that far better results were obtained by using 2 : 4-dinitro-

phenylhydrazine, which is very easily prepared by the method given by Allen [1930]. This substance is used in the form of a solution of 0·5 g. in 30 cc. 2*N* hydrochloric acid. No precipitation of the reagent occurs on adding to water. The method employed is essentially the same as that described by Neuberg and Kobel [1930]. The characteristics of the derivatives of this compound are also given in Table II. The acetaldehyde hydrazones are included in addition, in view of the fact that Neuberg and Gottschalk [1925] have reported the occurrence of acetaldehyde in the course of muscle metabolism in the presence of sulphite. As will be mentioned later, we have not been able to demonstrate the presence of acetaldehyde in any amount comparable with that of pyruvic acid or methylglyoxal in any of our experiments. We have employed Rimini's [1904] test for acetaldehyde, but the reaction obtained would correspond to the presence of only extremely small amounts of this substance.

Table II.

Reagent	Pyruvic acid hydrazone			Methylglyoxal osazone			Acetaldehyde hydrazone		
	Colour of precipitate	Reaction with alcoholic potash (6 %)	M.P.	Colour of precipitate	Reaction with alcoholic potash	M.P.	Colour of precipitate	Reaction with alcoholic potash	M.P.
p-Nitrophenylhydrazine	Yellow	Cherry-red	223–225°	Red*	Deep blue	302–304°	Yellow	Brown-red	128°
2 : 4-Dinitrophenylhydrazine	Lemon-yellow	Deep red	214°	Light red†	Blue-violet	298°	Yellow	Deep red	147°

* Recrystallised from nitrobenzene and heated for some time at 140°.
† Recrystallised once from pyridine and once from nitrobenzene.

A useful confirmatory test, though in itself anything but conclusive, for the presence of pyruvic acid and methylglyoxal is the iodoform reaction. Iodoform is rapidly produced in the cold by both methylglyoxal and pyruvic acid on the addition of alkali and iodine.

We have also employed the capacity of these compounds for binding sulphite as a method of estimating roughly the amounts formed. The procedure is described by Cook [1930]. It is possible by this method to estimate both the pyruvic acid and the methylglyoxal in a single sample. The total bound sulphite is estimated, the solution then being made acid and the methylglyoxal distilled off into sulphite and titrated in the distillate.

Experiments on rabbit muscle.

When working with frog muscle one is necessarily limited with respect to the amount of muscle it is possible to employ and hence the quantity of metabolic products which it is possible to detect or isolate. In order to overcome this limitation it was thought desirable to attempt to use rabbit muscle. The use of mammalian muscle for experiments of this nature is attended with several disadvantages, prominent among which are its comparatively rapid

deterioration when excised, either at the temperature of the animal or at lower temperatures, and the impossibility of ensuring that the tissue shall be permeated by any constituent of the fluid in which it may be immersed. We therefore decided to try minced rabbit muscle, although from the outset we realised fully the unphysiological condition of the tissue, and the fact that mincing or chopping destroys the power of the muscle to resynthesise carbohydrate.

The muscle was taken from the hind limbs and back of a rabbit killed by a blow on the head and bled from the heart. It was immediately minced, weighed, and apportioned out into beakers containing the various solutions prepared to receive it. After thorough mixing and disintegration of the chopped mass, the contents of the beakers were thoroughly aerated with oxygen, bubbled through for the duration of the experiment, which as a rule lasted for several hours at room temperature. At intervals nitroprusside tests were performed on about 3 cc. of the mixed tissue and fluid.

The details of the first of these experiments are as follows. Beaker A (control), 250 cc. phosphate buffer (p_H 7·2), 100 g. minced muscle; Beaker B (with sulphite), 242·5 cc. phosphate buffer, 7·5 cc. *M* Na_2SO_3, 100 g. minced muscle. These were oxygenated at room temperature for $3\frac{1}{2}$ hours. At the end of this time the contents of both beakers were strained through muslin and deproteinised by the addition in each case of 9 cc. of 50 % trichloroacetic acid. After filtering, the fluids were concentrated on a water-bath to about 20 cc. When cool, a few drops of an acetic acid solution of *p*-nitrophenylhydrazine were added to each, and the precipitates obtained were filtered off and washed. It was noted that the yield of solid from B was considerably the greater of the two.

The results are embodied in Table III.

Table III.

Nitroprusside reactions.

Time after commencement of exp. (mins.)	A (control)	B (with sulphite)
15	+ + +	+ +
40	+ +	+ + + +
65	+ +	+ + + +
210	+	+ + + +

Colour reactions of hydrazones with alcoholic potassium hydroxide.

A	B
Dirty bluish brown, similar to the colour obtained with excess of reagent	Strong deep red. Indicative of pyruvic acid

It is obvious that although towards the beginning of the experiment pyruvic acid was present to about an equal extent in both mixtures, it rapidly disappeared in the control, but on the other hand increased in concentration in the presence of the fixative.

Although the occurrence of pyruvic acid in minced rabbit muscle was thus

demonstrated, there was of course nothing to indicate that its presence was in any way dependent upon any oxidative process, in spite of the strongly aerobic conditions. The next step was to repeat the experiment under both aerobic and anaerobic conditions. To this end mixtures were set up as follows:

A. 50 g. minced muscle, 200 cc. buffer (p_H 7·2)	Aerobic	C. As A — Anaerobic
B. 50 g. minced muscle, 194 cc. buffer, 6 cc. M Na_2SO_3	Aerobic	D. As B — Anaerobic

A and B were oxygenated as before in beakers, while C and D were placed, together with 1 cc. of $M/10$ potassium cyanide solution, in Büchner flasks, which were evacuated. Nitroprusside tests were done from time to time over a period of about 4 hours. At the end of this time, 5 cc. of each mixture were ground with sand and the filtrates used for iodine titrations for the purpose of getting a rough idea of the relative amounts of bound sulphite. (In the cases of A and C a little sulphite was added to the liquid before titration.) The main bulk of the contents of the vessels was filtered through muslin and made up to 250 cc., including the addition of 20 cc. of 50 % trichloroacetic acid. The filtrates from this were concentrated to about 25 cc. and after cooling, 4 cc. of 2 : 4-dinitrophenylhydrazine solution in HCl added to each.

Table IV gives the complete results of this experiment.

Table IV.

Nitroprusside reactions.

Time after commencement of exp. (mins.)	A	B	C	D
40	–	–	–	+
175	+ –	+	–	+ +
240	–	+ +	–	+ + –
300	–	+ +	+ –	+ +

Iodine titrations for bound sulphite.

(5 cc. filtrate before deproteinisation was used; strength of iodine was 0·008 N.)

A	B	C	D
0·15 cc.	1·05 cc.	0·10 cc.	1·10 cc.

Extent to which precipitation occurred on addition of reagent.

	A	B	C	D
10 mins. after adding reagent	–	+ +	+ –	+ +
90 mins. after adding reagent	+	+ + +	+ +	+ + +

Colour reactions of hydrazones with alcoholic potash.

A	B	C	D
Blue-violet. Indicative of methylglyoxal	Deep red. Indicative of pyruvic acid	Blue-violet. Indicative of methylglyoxal	Deep red. Indicative of pyruvic acid

Here again it is seen that, in the presence of sulphite, pyruvic acid accumulates in quantities much greater than are found in the absence of the fixative. The colours given by the precipitates with 2 : 4-dinitrophenylhydrazine correspond exactly with the distribution of pyruvic acid as evinced by the nitroprusside tests. It is interesting also to note that in the absence of sulphite

methylglyoxal seems to preponderate over pyruvic acid. But the most important fact brought to light by this experiment is that in the presence of sulphite, the imposition of either aerobic or anaerobic conditions seems to make no difference to the appearance of pyruvic acid. This is of great significance, indicating as it does that the occurrence of pyruvic acid is not necessarily associated with oxidative processes, since these are excluded in C and D.

One explanation that suggests itself is that pyruvic acid (and methylglyoxal) may be intermediaries in the formation of lactic acid; indeed it seems difficult to place any other interpretation upon the results. If this is so, it should be possible to detect pyruvic acid among the substances present in an aqueous muscle extract during glycolysis. The next experiment to be done was then the following.

A. 100 cc. muscle extract
5 cc. phosphate buffer (p_H 7·2) (ten times strength)
3 cc. *M* Na_2SO_3
15 cc. 2 % glycogen
5 cc. water
} Oxygenated

B. 100 cc. muscle extract
5 cc. buffer (× 10)
3 cc. *M* Na_2SO_3
15 cc. water
5 cc. *M*/30 KCN
} Anaerobic

C. 100 cc. muscle extract
5 cc. buffer (× 10)
3 cc. *M* Na_2SO_3
15 cc. 2 % glycogen
5 cc. *M*/30 KCN
} Anaerobic

The muscle extract was prepared from rabbit muscle in the manner described in a former paper [Case, 1929]. Nitroprusside tests were performed as usual at intervals, and at the conclusion all the fluids were precipitated with 10 cc. of 50 % trichloroacetic acid, filtered, concentrated and treated with *p*-nitrophenylhydrazine. Table V sets forth the results.

Table V.

Nitroprusside reactions.

Time from commencement of exp. (mins.)	A	B	C
3	–	–	–
60	–	–	–
120	–	+	+
345	+	+	+ + +

Colour reactions of p-*nitrophenylhydrazones* (*-osazones*) *with alcoholic potassium hydroxide.*

A	B	C
Purplish-blue. Indicative of methylglyoxal with slight admixture of pyruvic acid	Deep blue. Indicative of methylglyoxal	Deep red, with very slight purplish tinge. Indicative of pyruvic acid, slightly contaminated with methylglyoxal

In this experiment, we find once more an appearance of pyruvic acid under anaerobic conditions, though not so marked quantitatively as in the case where the minced tissue was present in the system. Moreover, the addition of glycogen (in C) has caused a rise in the amount of pyruvic acid formed. As up to the present no attempt has been made to repeat this, we hesitate to

place much reliance on the result; but the fact, if confirmed, that added glycogen does have this effect seems to support the possibility that pyruvic acid is concerned in the process of glycolysis.

An experiment was now carried out with the object of ascertaining whether the presence of added lactate in the solution would have any similar effect. If pyruvic acid is being formed during the oxidation of lactic acid it might be expected that this would be the case.

A. 70 g. minced muscle
12·5 cc. buffer (× 10)
3·25 cc. *M* Na_2SO_3
Water to 125 cc.

B. 70 g. minced muscle
12·5 cc. buffer (× 10)
3·25 cc. *M* Na_2SO_3
10 cc. *M*/10 sodium lactate
Water to 125 cc.

} Aerobic, at room temperature

C. As A
D. As B
} Aerobic, in incubator at 35°

Table VI.

Nitroprusside reactions.

Time after commencement of exp. (mins.)	A	B	C	D
0	+ +	+ +	+ +	+ +
110	+ + +	+ + +	+ + +	+ + +
180	+ +	+ +	+ +	+ +
240	+ +	+	+	+

No hydrazones were prepared in this case, as the filtrates were used for other purposes, but it is obvious that the addition of lactate has not brought about any increase in amount of pyruvic acid, such as was observed when glycogen was added.

Up to this point attention has been concentrated mainly upon the detection of pyruvic acid. The demonstration of the presence of methylglyoxal is a more difficult matter, apart from the isolation of the osazone, since no rapid test exists for its identification in solution, corresponding to the Simon and Piaux reaction for pyruvic acid. In order to effect a convenient separation of methylglyoxal from pyruvic acid we resorted in several experiments to the procedure of which the ensuing is a typical example.

A flask was set up containing 25 cc. buffer (× 10), 6·5 cc. *M* Na_2SO_3, water to 250 cc. and 100 g. of minced muscle. This was allowed to incubate at room temperature for 5 hours. The nitroprusside reaction was then positive for pyruvic acid. The mixture was filtered through muslin, precipitated with trichloroacetic acid and again filtered. 20 cc. of 10 % sulphuric acid were added, and the whole of the fluid was distilled for an hour, the distillate being

Table VII.

	Product of interaction of 2 : 4-dinitrophenylhydrazine with	
	Residue in flask	Distillate
Colour of precipitate	Light yellow	Orange-red
Colour reaction with alcoholic potash	Deep red	Blue-violet

received in 10 cc. of 1 % sodium bisulphite cooled in ice. After cooling, both the residue in the flask and the distillate were treated with 2 : 4-dinitrophenylhydrazine (Table VII).

This shows that methylglyoxal has been distilled over, leaving pyruvic acid behind.

Attempts to demonstrate the presence of acetaldehyde in the distillate, where if formed it would be found, met with no success. The Rimini test was negative, and the characteristics of the hydrazones were not consistent with the presence of acetaldehyde (see Table II). If present at all, it can be only in very small amounts as compared with the amounts of methylglyoxal and pyruvic acid. Nitroprusside tests were strongly positive for the residue in the distilling flask, and negative for the distillate. Both fractions gave strongly positive iodoform reactions. In the case of a control experiment in which no sulphite was added, practically no nitroprusside test was given, and the iodoform reaction was only faintly positive.

It was considered desirable to perform a large-scale experiment with a view to isolating the derivatives of the products in sufficient quantity to carry out not only colour reactions but also melting-point determinations, in order to place their identification beyond question. The mixture employed consisted of 50 cc. phosphate buffer (× 10), 25 cc. *M* Na_2SO_3, water to 1 litre, 463 g. minced muscle. This was incubated at room temperature for 5 hours, at the end of which period a very strong nitroprusside test for pyruvic acid was given. The mixture was filtered through linen, using a press. Just over 1000 cc. of filtrate was obtained, and this was deproteinised with 100 cc. of 50 % trichloroacetic acid and filtered. The clear filtrate was again tested with nitroprusside and still gave a strongly positive reaction. It was then concentrated at about 40° *in vacuo* to a volume of 150 cc., more sulphite being added to ensure that the methylglyoxal and pyruvic acid should remain bound. After cooling, 40 cc. of 2 : 4-dinitrophenylhydrazine in HCl were added, the mixture was heated for a few minutes on a boiling water-bath and allowed to stand for some hours[1]. A fairly bulky orange precipitate was obtained, which was filtered off and washed with a little 0·5*N* HCl. The precipitate was treated with warm 25 % sodium carbonate, which dissolved the pyruvic acid derivative giving a dark-brown solution [Neuberg and Kobel, 1930]. On acidifying this solution with HCl, the hydrazone of pyruvic acid separated out as a lemon-yellow solid, which was further purified by re-solution in sodium carbonate and re-precipitation with acid, and finally by crystallisation from ethyl acetate.

Meanwhile the residue from the first sodium carbonate extraction, containing the methylglyoxal derivative, was washed once more with hot sodium carbonate to remove traces of the pyruvic compound, then with hot 2*N* HCl, and lastly with boiling water. It was then dried with a small quantity of

[1] When sulphite is present, this heating is frequently essential in order that hydrolysis of the sulphite-complex may be effected. If it be omitted, precipitation may be delayed or even suspended altogether.

alcohol and ether and recrystallised from pyridine; a dark red crystalline mass was obtained which on further crystallisation from nitrobenzene appeared as light red glistening crystals. These were washed with alcohol and ether. Both the pyruvic acid hydrazone and the methylglyoxal osazone were dried for 24 hours in a vacuum desiccator.

Previously to this, specimens of the hydrazone and osazone had been prepared from actual pyruvic acid and from a solution of methylglyoxal (made according to Neuberg and Hofmann [1930]), and had been purified in the same way.

Melting-points:

(*a*) Pyruvic acid 2 : 4-dinitrophenylhydrazone (from pyruvic acid) Neuberg and Kobel [1929] give 216°, Allen [1930] gives 213°	214°	(uncorr.)
(*b*) Pyruvic acid 2 : 4-dinitrophenylhydrazone (from muscle) ...	214°	,,
(*c*) Mixed melting-point	214–215°	,,
(*d*) Methylglyoxal 2 : 4-dinitrophenylosazone (from methylglyoxal) Neuberg and Kobel [1928] give 298°	297·5°	,,
(*e*) Methylglyoxal 2 : 4-dinitrophenylosazone (from muscle) ...	297°	,,
(*f*) Mixed melting-point	296°	,,

From a study of the foregoing Tables, notably III and VI, it will be observed that pyruvate, as demonstrated by the nitroprusside test, is sometimes to be met with in the solutions during the very early periods of the incubation. Where sulphite is present the quantity of pyruvate tends to increase as the incubation proceeds, but in the absence of sulphite the pyruvate progressively disappears. If this compound is indeed a transitory phase of lactic acid production, it is to be expected that immediately after a treatment such as mincing, which is strongly provocative of rapid glycolysis, the system will contain relatively large quantities of pyruvic acid. It is also apparent from the Tables that in the absence of a fixative this pyruvate very quickly disappears. It was therefore deemed of interest to try the effect of the addition of pyruvate to minced muscle both in presence and absence of sulphite, and the following experiment was accordingly performed.

A. 120 g. minced muscle
25 cc. buffer (× 10)
5 cc. 1 % potassium pyruvate
Water to 250 cc.

B. 120 g. minced muscle
25 cc. buffer (× 10)
5 cc. 1 % potassium pyruvate
6·5 cc. *M* Na_2SO_3
Water to 250 cc.

The experiment was aerobic. Nitroprusside tests were carried out as usual at intervals, the first test being made as soon as possible after setting up the mixtures. This time an attempt was made to render the tests to some extent quantitative.

For each test 5 cc. of the muslin filtrate were precipitated with 1 cc. of 50 % trichloroacetic acid. After filtering, 1 cc. of the clear solution was taken for estimation. The reagents employed were:

(*a*) 40 % acetic acid;

(*b*) freshly prepared 1 % aqueous sodium nitroprusside;

(*c*) ammonia (sp. gr. 0·880) diluted with an equal volume of water and saturated with ammonium sulphate.

The 1 cc. of solution to be tested was mixed with 0·5 cc. of (*a*), 3·0 cc. of (*b*) and 1·5 cc. of (*c*) in the order named.

Simultaneously, standards were set up consisting of 1 cc. of various dilutions of potassium pyruvate. These standards were prepared by mixing 5 cc. of a solution containing 2 mg. pyruvic acid in 5 cc. with 1 cc. of 50 % trichloroacetic acid, taking 1 cc. of this final mixture and adding the reagents as described above. The standard designated as I thus contained 0·34 mg. pyruvic acid in 1 cc. Standard II was half of this concentration, while standard III was one-quarter, and standard IV one-eighth.

The strength of the blue colours obtained in the unknowns was compared visually with the standards, a colorimeter not being employed.

Table VIII.

Time after commencement of exp. (mins.)	A	B
2	Negative (<IV)	Midway between I and II
30	Negative (<IV)	Slightly >I
90	Negative (<IV)	>I
240	Negative (<IV)	Considerably >I

Since 250 cc. of the original fluid contained 50 mg. pyruvic acid, the 1 cc. taken for testing would contain 0·17 mg. of pyruvic acid originally added. The above Table, approximate only though it is, shows therefore that (*a*) within a few minutes of adding the muscle there is a considerable increase in pyruvic acid in the case of the experiment with sulphite, and the value rises ultimately to more than twice the original figure, and (*b*) in the experiment without sulphite, all the added pyruvate has disappeared from the solution within 2 minutes.

It is hoped in future work to make this test more exactly quantitative, and experimentation is being carried out with this end in view.

As an additional check upon the information afforded by the above figures, the residual fluid in B (190 cc.) was treated with 2 : 4-dinitrophenylhydrazine in the usual way and after extraction with 25 % sodium carbonate and reprecipitation with HCl, to free it from the methylglyoxal derivative, was weighed. At the same time 190 cc. of a solution of the same composition as B, to which no muscle had been added, was similarly treated and the precipitate weighed.

Weight of 2 : 4-dinitrophenylhydrazone from B	0·222 g.
,, ,, ,, from solution of same composition	0·107 g.

This furnishes confirmation of Table VIII.

Experiments on frog muscle.

Although at the inception of this work frog muscles were exclusively used, and rabbit muscle was not employed till later on, it has been found more

convenient for the purposes of this paper to describe these latter experiments before those which are about to be reported. The methods used for detecting and identifying pyruvic acid and methylglyoxal are identical in the two series, and hence need not be further described in detail.

In all the experiments reported below, when muscles are referred to as "stimulated," the technique consisted in immersing the limbs in the appropriate solutions in small beakers, and, by means of a small induction coil, stimulating indirectly *via* the nerves. It has since been found more effective to apply direct stimulation, as in the presence of compounds such as sulphite the nerve seems to lose its irritability much sooner than does the muscle. Those portions of wire actually in contact with the muscle were always of silver. Where muscle is described as "chopped" it was dissected from the bone and chopped fairly finely with scissors. In aerobic experiments oxygen was bubbled through the solutions bathing the muscles, while in anaerobic ones the beakers containing the muscles were enclosed in a large vacuum desiccator on the bottom of which was a layer of alkaline pyrogallol, while the desiccator itself was filled with nitrogen by repeated evacuation and replenishment from a nitrogen cylinder. The wires for stimulation in such cases were led out through the rubber stopper.

It will be best, for the sake of brevity, to present the whole series of experiments in a consecutive manner and then discuss the significance of the results.

Exp. I.

A. 20 cc. frog Ringer solution (× 10)
20 cc. phosphate buffer, p_H 7·2 (× 10)
Water to 200 cc.

B. As A, with the addition of 5 cc. *M* Na_2SO_3

Two limb-pairs, severed at the girdle, were placed in each and were stimulated as long as irritability lasted. The experiment was aerobic and continued for 5 hours. The muscles were then ground with sand and water (15 cc.). Nitroprusside tests were done on the muslin filtrates.

A	B
–	+ + + +

Exp. II.

A. 5 cc. frog Ringer solution (× 10)
5 cc. buffer (× 10)
5 cc. *M*/10 sodium lactate
Water to 100 cc.

B. As A, with the addition of 3 cc. *M* Na_2SO_3

In each beaker were placed the muscles from three limb-pairs, dissected carefully from the bones, but not chopped. Oxygen was bubbled for 4 hours, after which the muscles were ground with sand and trichloroacetic acid.

The nitroprusside reactions were negative for pyruvate in both cases.

The *p*-nitrophenylhydrazine derivatives were prepared; B yielded about twice as much as A. After washing, these both gave a strong pure blue colour

with alcoholic potassium hydroxide, indicating that the product was in each case methylglyoxal *p*-nitrophenylosazone.

Exp. III.

A and B. 10 cc. buffer (× 10)
10 cc. frog Ringer solution (× 10)
5 cc. *M*/10 sodium lactate
2·5 cc. *M* Na_2SO_3
Water to 100 cc.

One limb-pair was placed in each, the only difference being that the mucles in A were stimulated, while those in B were not. The experiment was aerobic and lasted for 5 hours. The muscles were then ground with sand and 15 cc. of their respective fluids.

Nitroprusside reactions:

A	B
++++	+

The remainder of the filtrates were deproteinised with trichloroacetic acid, filtered, concentrated, and treated with *p*-nitrophenylhydrazine.

Colours with alcoholic potassium hydroxide:

A	B
Deep red. Indicative of pyruvic acid	"Mixed" colour, with preponderance of blue. Indicative of methylglyoxal, slightly contaminated with pyruvic acid

Exp. IV.

A.	B.
10 cc. buffer (× 10)	10 cc. frog Ringer solution (× 10)
3 cc. *M* Na_2SO_3	3 cc. *M* Na_2SO_3
Water to 100 cc.	Water to 100 cc.
Chopped muscle from one limb-pair	One intact limb-pair, not stimulated

The experiment was aerobic and lasted for 6 hours. Both lots of muscle were then ground with sand and water.

Nitroprusside reactions:

A	B
−	++++

The remainder of the filtrates were deproteinised and treated with *p*-nitrophenylhydrazine.

Colours with alcoholic potassium hydroxide:

A	B
Deep blue. Indicative of methylglyoxal	Deep red. Indicative of pyruvic acid

Exp. V.

50 cc. frog Ringer solution (× 10)
12·5 cc. *M* Na_2SO_3
0·25 g. $NaHCO_3$
Water to 500 cc.

A. Two limb-pairs unstimulated in 100 cc.
B. Two limb-pairs stimulated in 100 cc.
C. Muscle removed from 4 legs (not chopped) in 50 cc.
} Aerobic

D. Two limb-pairs unstimulated in 100 cc.
E. Two limb-pairs stimulated in 100 cc.
F. Muscle removed from 3 legs (not chopped) in 50 cc.
} Anaerobic

The limbs were chosen so that the weight of muscle in each vessel was approximately the same (about 8 g.). The duration of the experiment was 2 hours; the muscles were then ground with sand and 15 cc. of water.

Nitroprusside reactions:

A	B	C	D	E	F
+	+ + +	+	+ +	+ + + + +	+

Iodine titrations for bound sulphite (on 2 cc. of filtrate from ground tissue, and 5 cc. of fluids in vessels). Strength of iodine 0·008 *N*.

	A	B	C	D	E	F
Fluid	0·10	0·15	0·91	0·15	0·20	0·55
Tissue	3·60	3·60	2·80	4·30	4·60	2·50

These iodine figures bear out the results of the nitroprusside tests in that in D and E (the anaerobic whole limbs) the bound sulphite is greater in amount than in the corresponding aerobic limbs A and B.

They show also that in the case of intact muscles diffusion of the metabolic products into the surrounding fluid is almost negligible.

The main facts emerging from this series of experiments, which is of necessity as yet far from complete, are as follows.

(i) Stimulation always leads to an increased amount of pyruvic acid in the tissue (Exps. I, III, V).

(ii) Pyruvic acid can be demonstrated to occur even in the absence of sulphite (Exp. I).

(iii) Aerobic conditions are not essential for the production of pyruvic acid, which indeed on the other hand appears to be favoured by anaerobiosis (Exp. V).

(iv) As with rabbit muscle, methylglyoxal can also be detected (Exps. II, III, IV), and in presence of sulphite seems to be fixed to a greater extent (Exp. II). This compound appears to be produced under conditions which are not yet determined, but it is noteworthy that in cases where the muscle has been removed from the limbs, as in Exp. II, methylglyoxal always seems to occur rather than pyruvic acid. This has frequently been found to be the case in other similar experiments not reported here, but at present we have no idea of what the significance of this circumstance may be.

At all events, on the whole the experiments with frog muscle confirm strikingly the results obtained with rabbit muscle. The favourable influence of stimulation upon pyruvic acid formation is evidence for the intermediary occurrence of this substance during lactic acid production, as is also the absence of any sign of inhibition of its formation under anaerobic conditions.

Discussion.

The results recorded in this paper are to be regarded as being of a preliminary nature only. Much more work will have to be accomplished before

the great technical difficulties associated with a problem of this kind can be surmounted to such an extent as to render the results quantitative.

We believe that we have fairly conclusively shown that both pyruvic acid and methylglyoxal do participate at any rate in some process or other occurring in muscle. With regard to the factors conditioning their appearance or non-appearance there is practically nothing that can at present be said with any degree of certainty. There can be no doubt that these substances in their relation to one another constitute part of a very delicately balanced system, the equilibrium of which is likely to be disturbed by seemingly slight variations in the surrounding medium and conditions. This relation, it is intended, will form the subject of further research.

That pyruvic acid is of importance in the metabolism of muscle is strongly suggested by the recent work of Toeniessen and Brinkmann [1930], who demonstrated a rapid removal of this substance when perfused through the musculature of a rabbit. Our own results with minced muscle confirm this rapid disappearance.

It has long been a widely-held view that methylglyoxal occupies an important position in the series of changes resulting in lactic acid production in muscle, and our results are in accord with this also.

Very many fresh experiments suggest themselves, which we propose to make the subject-matter of a future communication. Among these may be mentioned a study of pyruvic acid formation in muscle poisoned with iodo-acetic acid, with fluoride and with amylase. The bearing of such experiments upon the question as to whether pyruvic acid and methylglyoxal are indeed being formed during the change carbohydrate → lactic acid is obvious.

The possibility must not be overlooked that lactic acid may be oxidised to pyruvic acid anaerobically in the presence of hydrogen acceptors in the muscle, or even that the source of the pyruvic acid may not be carbohydrate in nature at all, but that for instance this compound may be an intermediary in fat metabolism. On the whole, however, we are not inclined to regard either of these hypotheses as very probable.

Summary.

1. The technique of the use of sodium sulphite as a fixative has been employed for investigating some of the substances concerned in the intermediary metabolism of muscle.

2. Pyruvic acid and methylglyoxal have been shown to occur in frog and rabbit muscle, both in the presence and absence of sulphite, and under both aerobic and anaerobic conditions. These substances have been isolated and identified as the 2 : 4-dinitrophenyl-hydrazone and -osazone respectively.

3. In view of the conditions, in so far as they have been established, under which these compounds make their appearance, it is suggested that they may both be intermediary between carbohydrate and lactic acid.

It is with much pleasure that we express our thanks to Sir F. G. Hopkins and Prof. J. B. S. Haldane for their interest in this work.

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CXLV. PIGMENTATION OF THE LIVER OF THE MONK (OR ANGLER) FISH.

By JOHN ARNOLD LOVERN and RICHARD ALAN MORTON.

From the Torry Research Station, Aberdeen, and The University, Liverpool.

(*Received July 3rd, 1931.*)

In studying the livers of various species of fish, one of us (J. A. L.) noticed the frequent occurrence of a pronounced pink pigmentation in the interior portions of the liver of the monk fish (*Lophius piscatorius*). The livers of gadoid fish, such as codling, hake, *etc.*, are of uniform colour throughout, being in most cases almost white. Colouring is sometimes seen in the Elasmobranchii (*e.g. Squalus acanthias* and *Raia clavata*) but as a rule the livers are very pale. The exceptions are mottled in appearance, and the pigmentation is due to a grey-black substance, which is not extracted along with the oil.

The pigmentation of monk-livers is, therefore, a distinct and unusual phenomenon. The colouring matter is localised in irregularly shaped deposits found much more often in the interior than in the exterior portions of the liver. The intensity of the pigmentation varies between wide limits, not only from fish to fish, but also for the localised deposits in a particular liver. In an absolutely fresh liver the intensity of the pigment is much less than in the same liver after exposure to air, and oxidation thus appears to play a part in its formation.

Histological examination of the pigmented areas shows that not all the cells are pigmented, even in such areas. The distribution of pigment appears to be quite irregular. Under high power the pigment is seen to be present in the form of deep orange droplets (possibly dissolved in oil), of various sizes, but of the same order of magnitude as the liver cells. It is worth noting that the formaldehyde fixative used did not appreciably bleach the pigment. Bleaching experiments with a strong solution of ferric chloride in 50 % alcohol, in which pigmented histological sections were immersed, revealed a fairly rapid and complete bleaching. Two examples may be given.

Section from liver No. 1.

Pigmented section after 5 minutes in $FeCl_3$	No appreciable bleaching
Same section after 20 minutes in $FeCl_3$	Complete bleaching of pigmented areas

Section from liver No. 2.

Pigmented section after 5 minutes in $FeCl_3$	Some bleaching
Same section after 20 minutes in $FeCl_3$	Mainly bleached
Same section after 30 minutes in $FeCl_3$	Completely bleached

The pigment accompanies the oil when the livers are extracted by means of solvents. It is noteworthy that oil cannot be obtained from these livers by steaming, although the oil content is frequently as much as 50 %. In one experiment a small portion of the unsaponifiable matter was prepared and found to contain much of the pigment present in the original oil. This unsaponifiable matter (mainly cholesterol) was spread in a thin film on a glass slip, and exposed to air and sunlight for some days. Bleaching was only gradual, over a week elapsing before the colour had all disappeared.

The nature of this pigment has not yet been fully elucidated, and the investigation is being continued in the hope of isolating a quantity sufficient for identification. Some interesting properties have, however, already emerged. Previously to commencing the study of monk-livers it had been found that oils prepared from the various parts of any particular fish-liver gave the same intensity of blue colour with antimony trichloride. In the case of the first three monk-livers examined, however, the following results were obtained (J. A. L.).

		Blue value (Carr-Price)
Liver (1)	Oil from pigmented interior	9·6
	Oil from non-pigmented exterior	3·6
Liver (2)	Oil from pigmented interior	4·4
	Oil from non-pigmented exterior	2·4
Liver (3)	Oil from pigmented interior	14·0
	Oil from non-pigmented exterior	3·2

In view of the well-known chromogenic properties of carotenoids it appeared worth while to investigate whether the pigment was carotenoid in type, especially since in the case of a monk-liver which apparently contained no pigment at all, the oils from the inside and outside layers gave the same intensity of blue colour. However, in later experiments it was found that this difference between the chromogenic power of the oils from the pigmented and non-pigmented parts is not always observed. Thus, in the case of a liver having a very intense localised pigmentation, the oils from the red and white parts of the liver respectively gave the same blue colour in the antimony trichloride test, namely 2·8 Carr-Price units. The intensities of the blue colours were thus not proportional to the redness of the oils.

It was decided to examine the oils spectroscopically in order to see whether additional evidence as to the nature of the coloured substance could be obtained. The following samples were studied.

Sample (A). The mixed oils from livers (1), (2) and (3). (These were unfortunately mixed for other purposes before experiments on the nature of the pigment were undertaken.)

Sample (A′). The unsaponifiable matter from A, dissolved in chloroform.

Sample (B). Oil from the inside of a monk-liver.

Sample (C). Oil from the outside of the same liver exhibiting little or no pigmentation.

Sample (D). Oil from non-pigmented parts of a liver with deeply pigmented deposits.

Sample (E). Oil from pigmented parts of the same liver.

Sample (F). Ether-extracted oil from several complete livers exhibiting a fairly uniform distribution of a rather low intensity of pigmentation.

All the oils showed the band at 328 $m\mu$ as a faint inflexion, superimposed on much more intense general absorption.

Samples (A), (E) and (F) gave bands with maxima at 480–485 $m\mu$, 450–457 $m\mu$ and 422–430 $m\mu$, in the same region and of the same type as those given by carotenoid pigments such as carotene or xanthophyll. At the same time the maxima do not agree exactly with those of carotene dissolved in an oily medium (*e.g.* arachis oil). Identification of definite members of the carotenoid group is a difficult matter, especially in the presence of vitamin A,

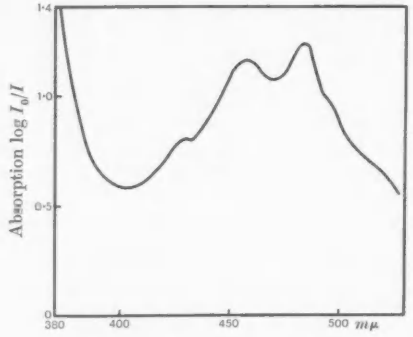

Fig. 1. Monk-liver oil F_1. $\frac{1}{2}$ mm. layer.

so that although the evidence suggests a carotenoid, it is by no means conclusive. It may be significant that materials containing the pigment (both in oils and extracts of the unsaponifiable matter dissolved in ether or chloroform) respond to many of the colour tests for carotenoids, *e.g.* with strong sulphuric acid there is a gradual development of an intense green colour, which, although gradually fading, persists for 24 hours. There is some doubt as to the product of the interaction of the pigment and antimony trichloride. The blue solution obtained with carotene exhibits a maximum near 590 $m\mu$, and this could not be detected, whilst the blue colour with monk-liver oil exhibited the two bands with maxima at 572 $m\mu$ and 606 $m\mu$ respectively associated with vitamin A. The presence of these maxima may mask absorption in the region of the 592 $m\mu$ band and render detection difficult.

It would thus appear that whilst some of the evidence suggests a carotenoid nature for the pigment, there is no satisfactory evidence as to any particular carotenoid—as stated above it is hoped to isolate the pure pigment in sufficient quantity for identification. If it should prove to be carotenoid in nature, it

may well provide additional evidence in the carotene-vitamin A problem. It is interesting to note that the vitamin A content of the livers of *Lophius piscatorius* is generally low compared with other species.

Experiments on monk-liver oils.

Oil	$E^{1\%}_{1\,cm.}$ 328 $m\mu$ (gross)	Bands in the visible spectrum	$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$
A	1·4	480, 450, 422 $m\mu$ ($\frac{1}{2}$ mm. oil itself)	1·0	0·55
Unsaponifiable matter	—	482, 452, 420, 400 $m\mu$	—	—
B	0·26	Ill-defined	0·24	0·2
C	—	Ill-defined	0·2	0·2
D	0·7	Inflexion only	0·42	0·12
E	0·85	485, 455, 422 $m\mu$ ($\frac{1}{2}$ mm. oil itself)	0·44	0·24
F	0·82	485, 455, 430 $m\mu$ ($\frac{1}{2}$ mm. oil itself)	0·825	0·25

Discussion.

The spectroscopic data on the monk-liver oils indicate that the vitamin A potency varies between 0·2 and 0·8 of that associated with an average cod-liver oil (1·0 spectroscopic unit or about 12–13 Carr-Price units). The particular samples studied all showed a clear maximum at 572 $m\mu$ in the antimony trichloride colour test, but the capacity to give the more usual 606 $m\mu$ maximum was latent [*cf.* Heilbron, Gillam and Morton, 1931]. The ultra-violet absorption band at 328 $m\mu$, characteristic of vitamin A appears to be present in the intensity to be expected from the colour test in all the oils, but owing to superimposed, more general absorption, the band only appears as a marked inflexion.

There appears to be no direct connection between the pigment and vitamin A, as the non-pigmented oils cannot be differentiated from the pigmented oils by any property other than colour.

The pigment resembles carotene in its absorption in the visible spectrum. Since 0·5 mm. layers of different oils exhibit the three bands at 480, 450 and 422 $m\mu$, the points of maximum absorption corresponding with

$$E = \log I_0/I = 0{\cdot}8 - 1{\cdot}2,$$

we have

$$E^{1\%}_{1\,cm.}\ 450\,m\mu = 0{\cdot}16 - 0{\cdot}24.$$

Now for carotene itself $E^{1\%}_{1\,cm.}$ 450–460 $m\mu$ = 1400, whilst the intensity of the blue solution obtained with carotene and antimony trichloride is of the order $E^{1\%}_{1\,cm.}$ 590 $m\mu$ = 300–400. Accepting the higher figure, we can calculate approximately the intensity of a blue colour due to the quantity of pigment present in monk-liver oil on the assumption that it is carotene. The value for $E^{1\%}_{1\,cm.}$ 590 $m\mu$ is 0·04 to 0·07. Now in the present series of monk-liver oils the values for $E^{1\%}_{1\,cm.}$ 572 and $E^{1\%}_{1\,cm.}$ 606 $m\mu$ are never less than 0·20. Hence we have

the rather important conclusion that, deeply pigmented as the monk-oils are, the quantity of vitamin A present even in oils having only one-fifth of the potency of cod-liver oil, is sufficient to give a blue colour from 3 to 6 times as intense as that to be expected from the pigment on the basis that it is a carotenoid. It is thus not very surprising that the pigment did not give rise to a distinct detectable band in the colour test.

There is now considerable evidence that carotene is heterogeneous, containing two or more closely related substances. From the present point of view this fact increases the difficulties in identifying the pigment of monk-livers. We are endeavouring to secure pigmented monk-liver oils which are either very poor in vitamin A or relatively very rich in pigment, but, as the conditions governing seasonal and other variations in potency and pigmentation are unknown, the test may well prove difficult.

SUMMARY.

Monk-livers often contain irregular deposits of a red fat-soluble pigment, hitherto not found in fish-livers. The pigment appears to act as a chromogen towards antimony trichloride, but quite small quantities of vitamin A are so intensely chromogenic as to obscure the rôle of the pigment. Such evidence as is available points to the pigment being carotenoid in type.

We are indebted to the Department for Scientific and Industrial Research for support granted to this investigation.

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CXLVI. THE MITTELMANN PROCESS FOR TREATING FISH-LIVERS.

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Two reports by Mittelmann on "Les graisses de la Morue et nouvelles méthodes de leur préparation" [1925] and "The manufacturing of fish conserves of Murman" [1927] are somewhat difficult of access, and this may account for the fact that certain distinctly important claims have so far neither been confirmed nor refuted in the literature. We are indebted to Bezssonoff [1929] for a summary of Mittelmann's work, the gist of which is as follows. Fresh cod-livers are sealed in evacuated tins which are then heated to 120°, the process amounting to autoclaving the livers. On opening the tins the liberated oil can readily be poured off, and it is said to be pleasant in taste and free from the typical disagreeable smell characteristic of all cod-liver oils prepared by any method used in the industry. "On testing the oil immediately after opening a tin no blue colour with antimony trichloride was obtained, but if the oil was re-examined after 10 days a positive colour test was recorded. At the same time, the characteristic unpleasant smell of commercial cod-liver oils began to manifest itself. Thus, contact with air causes the appearance of the evil-smelling substance (probably as a result of the formation of bodies with ethenoid linkages) and simultaneously brings into being the property of giving a blue colour with antimony trichloride."

The importance of these claims is considerable both from the theoretical and practical standpoints. If vitamin A is really present in the oils from freshly opened tins the antimony trichloride colour test must have serious limitations, to say the least. If, on the other hand, the colour test is definitely a valid measure of vitamin A, then since negative results are obtained with freshly opened tins, the oil from the autoclaving process must be devoid of vitamin A. As, however, the chromogenic property of the oil is acquired on standing in air, the vitamin must be an oxidation product of a precursor substance stored in the liver.

These implications made it necessary in our opinion to undertake a careful repetition of the work of Mittelmann. Accordingly arrangements were made for the livers of fish caught by Aberdeen trawlers to be canned and autoclaved at the Torry Research Station and the oils examined by the Lovibond technique applied to the antimony trichloride colour test. The tins were also to

be sent to Liverpool with the minimum delay so that a complete spectroscopic examination could be carried out. We are indebted to Mr John Bruce of Messrs Marshall and Co., Ltd., of Aberdeen for assistance in canning the livers. The tins used were lacquered on the inside to guard against contamination by the metal, the lacquer being chemically inert and oil-resisting. Tins of one pound capacity were used as these were the largest which could be sealed under vacuum. They were not large enough to contain whole livers, so that in all cases except those of small codling, the livers were cut into pieces before packing. After evacuating to 1 inch of mercury and sealing, the tins were autoclaved at 120° for 2 hours.

The first experiments were carried out on small codling caught off Aberdeen, and the livers were only about 4 hours old when canned. The oil obtained from the tins was examined the same day (19. ii. 1931) and gave a reading of 1·84 Carr-Price blue units (Lovibond). After storage under nitrogen for 6 days the value was 2·24 units, and after 13 days 2·28 units.

Ultra-violet absorption spectra for the oil from freshly opened tins, photographed less than 3 days after the fish were caught, showed that the codling-liver oils were remarkably transparent. With a 1 mm. layer of the oil itself (no solvent) 3 % of the incident light was transmitted at 299·5 $m\mu$, the curve showing a small maximum at 320–330 $m\mu$, the value for the gross uncorrected absorption at this point being E (= $\log I_0/I$) 0·125 reduced to the basis of a 1 % solution and a 1 cm. cell. With antimony trichloride a definite blue-violet colour was produced, the solution exhibiting a well-defined maximum at 572 $m\mu$. With most cod-liver oils the blue solution is characterised by a maximum at 606 $m\mu$, the spectral absorption curve being unsymmetrical owing to the presence of greater or smaller absorption at 572 $m\mu$, which is usually less intense than that at 606 $m\mu$. With the codling-liver oil from freshly opened tins, there was definite, measurable absorption at 606 $m\mu$, but this was approximately only half as intense as the absorption at 572 $m\mu$. On standing exposed to air, or on oxidation with oxygen at 70°, the capacity to give a blue colour with antimony trichloride underwent a considerable increase. This was reflected in the spectral absorption of the blue solution by an increase in absorption at 606 $m\mu$, which was not accompanied by any appreciable increase or decrease at 572 $m\mu$.

A number of larger codling-livers were also canned and autoclaved at Aberdeen and examined spectroscopically at Liverpool. Here again, the freshly opened tins yielded oils which exhibited a small absorption band at 328 $m\mu$ and gave a colour test in which the 572 $m\mu$ band predominated over the 606 $m\mu$ maximum. It is interesting to note that the blue solution with antimony trichloride slowly faded to a clear yellow colour characterised by a sharp maximum at 460–465 $m\mu$.

The preliminary experiments on codling-livers having given indications of an increase in the chromogenic value of the oils from autoclaved livers on standing, it was decided to repeat the experiments under more defined con-

ditions and with adequate controls. Accordingly ½ cwt. of cod-livers were obtained from fish landed at Buckie (3. iii. 1931) and gutted on shore. Nets had been out since 28. ii. 1931 so that many of the fish had been dead for a few days. The livers were conveyed to Aberdeen and each was cut into two roughly equal parts. One half of the catch was canned and autoclaved at 120°, whilst the other half was steamed with direct steam in the usual way for preparing cod-liver oil.

The Buckie cod were on spawn at the time of catching, whereas the codling already referred to were either immature or just about to spawn. It must be emphasised that the steamed liver oil was obtained from half the whole catch, whereas individual tins subjected to the Mittelmann process would be representative of two or three livers, so that the experiment could only afford a qualitative control, the well-known variations in potency of the oil from individual livers precluding strictly quantitative control.

Lovibond readings obtained at Torry Research Station on March 4th, 1931, gave readings of 4·0 and 6·4 Carr-Price units for the oils from autoclaved and steamed livers respectively. After 3 days' storage under nitrogen the oils gave values of 8·0 and 7·2 blue units. The oils and tins reached Liverpool on March 5th, and were examined on that and the following days. The spectroscopic examination of the antimony trichloride-oil solution gave results indicating that little or no change had occurred in transit. The blue solution showed a well-defined maximum at 572 $m\mu$ together with marked, but much less intense, absorption at 606 $m\mu$. The ultra-violet band with maximum near 328 $m\mu$ was shown very clearly, but at an intensity much greater than would be expected from the proportion of the chromogen responsible for the 606 $m\mu$ absorption in the colour test.

Different tins were opened at various times for several weeks after receiving the oils. It was found that in the sealed tins the oil gradually underwent a change which resulted in enhanced absorption at 606 $m\mu$, but within the limits of error no appreciable change occurred either in the absorption at 572 $m\mu$ or 328 $m\mu$ in the ultra-violet spectrum. It was at any rate clear that the marked predominance of the 572 $m\mu$ band slowly disappeared. Storage of the oil in bottles under nitrogen likewise failed to prevent a slow change resulting finally in a very marked increase in the 606 $m\mu$ band relatively to the 572 $m\mu$ band. Oxidation with oxygen at 70° or ozone at room temperatures likewise caused a marked increase in the 606 $m\mu$ band, which in this case was ascertainably not at the expense of a diminution either of the 572 $m\mu$ band or the ultra-violet band. It is noteworthy that the blue solution again faded to a bright yellow marked by a maximum at 465 $m\mu$.

A few comments on Mittelmann's process as a method for the manufacture of cod-liver oil are necessary. In our hands, the yield of oil is poor and variable, in some cases more water than oil being obtained. In nearly every case it was found necessary to dry the oil with anhydrous sodium sulphate before carrying out any tests. It is moreover very difficult to free the liver débris

from oil. Comparing the autoclaved liver oil with the steamed liver oil prepared from the same livers, there is no doubt that the steaming process can, at any rate in the laboratory, give oils superior in taste, odour and colour. The oil from the tins has a peculiar "meaty" taste and smell, quite different from, and perhaps worse than, the fishy taste of medicinal cod-liver oil. If the oils are kept for some weeks in bottles, or if the tins are not opened for some weeks, there is a very noticeable improvement in taste and smell. As regards colour, the oils are very pale, but not quite so good in this respect as the steamed oil obtained under laboratory conditions. The oil from the tins exhibits less tendency to deposit stearin than the steamed oils, and this is probably a consequence of the prolonged heating of the oil, as crude cod-liver oil deposits much less stearin after heating than before.

Drummond and Hilditch [1930] have expressed the view that vitamin A is not necessarily associated with the fat in the liver, the fat merely dissolving the vitamin on disruption of the cells. It may therefore be significant that the liver tissue does not appear to be so completely disintegrated by Mittelmann's process as by careful and adequate steaming.

Summary of data.

Oil	$E^{1\%}_{1\,cm.}$ 606 $m\mu$	$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 328 $m\mu$
Small codling-livers (autoclaved):			
(*a*) Freshly opened tins	0·06	0·12	—
(*b*) After 4 hours	0·06	0·12	0·125
(*c*) After 24 hours	—	—	0·16
Ditto, treated with oxygen for 5 mins. at 70°:			
(*a*) Examined at once	0·10	0·12	—
(*b*) Left overnight	0·18	0·12	—
Larger codling-livers:			
Freshly opened tins	0·13	0·20	—
" 4 hours later	0·13	0·20	0·22
Two more tins (freshly opened)	0·125	0·175	—
Oxidised	0·32	0·175	0·21
Steamed cod-livers: Fresh	0·35	0·76	0·73
Autoclaved cod-livers:			
Freshly opened tins	0·27	0·60	—
Two more freshly opened tins	0·30	0·63	—
Steamed cod-livers: 12 days old	0·60	0·78	0·75
Autoclaved cod-livers:			
10 tins opened 10 days after canning	0·39	0·72	0·70
9 days after opening	0·42	0·67	—
3 tins opened 3 weeks after canning	0·61	0·94	—
5 tins opened 3 months after canning	0·36	0·54	—

Discussion.

The most important result emerging from the work is the confirmation of Mittelmann's observation that the chromogenic value of fresh cod-liver oils sometimes increases on standing for a relatively short time. We have not so far encountered a cod-liver oil which failed to respond qualitatively to the colour test, but with codling-, cod- and monk-liver oils it is clear that the chromogenic values may increase on oxidation. Inasmuch as the experiments

with the steamed cod-liver oil indicated precisely the same tendency towards an increase in the blue value as the autoclaved oils, there is no evidence for regarding the Mittelmann process *per se* as having any special significance. Moreover, the ultra-violet band, which is at present perhaps the least debatable criterion of vitamin A, discloses no qualitative differences between autoclaved liver oils and oils prepared by other processes. The results agree well with the conception advanced in the following papers, that liver oils contain two distinct if not unrelated chromogens.

We are indebted to the Committee on Research of the University of Liverpool and to the Department for Scientific and Industrial Research for financial assistance towards the cost of this investigation.

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CXLVII. THE ANTIMONY TRICHLORIDE COLOUR TEST AND THE ULTRA-VIOLET ABSORPTION OF LIVER OILS AND CONCENTRATES.

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(*Received July 3rd, 1931.*)

In a recent paper [Morton, Heilbron and Thompson, 1931] it was stated that the blue solution obtained by the interaction of liver oils and antimony trichloride was liable to exhibit an absorption maximum at 572 $m\mu$, instead of, or in addition to, the maximum at 606 $m\mu$ which has hitherto been studied [Morton and Heilbron, 1928; Drummond and Morton, 1929]. It was also recorded that with concentrates a maximum at 583 $m\mu$ occasionally made its appearance with or without the simultaneous appearance of the more usual band at 620 $m\mu$. No wholly satisfactory explanation of these observations being forthcoming, it was decided to examine a large number of oils and concentrates as completely as possible with a view to re-testing the correlation between ultra-violet absorption [Morton and Heilbron, 1928] and the spectroscopic data on the colour test. For this purpose, the intensities of absorption at 572 $m\mu$ and 606 $m\mu$ were determined for the blue solutions, irrespective of whether one or two maxima were visually apparent in the eye-piece of the spectroscope.

From the results of Drummond and Morton [1929] it is clear that the blue colour with antimony trichloride and the ultra-violet band at 328 $m\mu$ both vary in intensity exactly as would be expected if both were due to vitamin A. The experimental error of the biological assay is however so great that although the correlation of these properties with the vitamin is highly probable, it is not proved in a rigid quantitative sense.

The ultra-violet band is however on a different footing from the blue colour. It provides a mechanism for the photochemical destruction of vitamin A and itself disappears on irradiation. The band moreover appears in the liver-fat of rats fed on a vitamin A-deficient diet with carotene as a growth-promoting supplement, such fat being itself potent in vitamin A. The band is however absent from the liver-fat of rats maintained on a vitamin A-free diet without added carotene [Moore, 1930; Drummond, Ahmad and Morton, 1930]. Similar considerations indicate that absence of chromogen coincides with absence of vitamin, and presence of chromogen coincides with presence of vitamin. This state of affairs would be not unsatisfactory were it evident that the chromogenic property of liver oils is due to a single, well-defined entity. The colour-

producing mechanism is however complicated and difficult to describe unambiguously. The displacement from 606 to 620 $m\mu$ shown by concentrates is troublesome in the sense that it lessens the precision of the description of the blue colour. It raises the question as to whether purer preparations of vitamin A would give rise to an enhanced displacement. Moreover the appearance of bands at 572 or 583 $m\mu$ in certain oils and concentrates can only mean an unusual variability of the colour substance or complex, or else the existence of a new chromogen in certain oils and concentrates. If a new chromogen can appear in a few oils, the possibility cannot be evaded that all oils may contain varying proportions of two chromogens.

As a first step in investigating this matter, attention may be drawn to the fact that if a series of oils is tabulated in order of increasing intensity of absorption at 328 $m\mu$, the absorption at 606 $m\mu$ should increase steadily down the table. Examination of Table I, which shows the data for 52 liver oils in

Table I. *Cod- and other fish-liver oils in which the value of* $E^{1\%}_{1\,cm.}$ *328* mμ *is less than the value of* $E^{1\%}_{1\,cm.}$ *606* mμ.

328 $m\mu$	606 $m\mu$	328 $m\mu$	606 $m\mu$
0·14	0·15	1·38	1·59
0·5	0·54	1·40	1·60
0·5	0·84	1·50	1·51
0·53	0·62	1·55	1·80
0·55	0·76	1·55	1·91
0·62	1·0	1·64	1·75
0·66	0·88	1·70	1·9
0·8	1·22	1·87	1·98
0·8	1·07	1·95	2·05
0·85	0·88	2·62	3·70
0·96	1·38	3·67	5·1
0·97	1·5	4·83	8·5
1·0	1·2	5·0	12·0
1·05	1·7	5·2	9·5
1·05	1·35	5·2	6·84
1·1	1·68	5·25	8·5
1·1	1·18	5·3	7·5
1·13	1·55	5·4	7·8
1·13	1·65	6·0	8·91
1·15	1·75	6·0	7·5
1·15	1·47	6·0	7·72
1·20	1·35	6·25	9·2
1·20	1·82	7·0	9·0
1·25	1·45	10·0	21·5
1·26	1·65	10·0	15·0
1·30	1·38	11·0	18·8

all of which the 606 $m\mu$ band is well shown, discloses the same general trend over the table as a whole, but many minor discrepancies. In Table II are given the data for 19 oils in which the intensity of the 606 $m\mu$ band is unusually low in relation to the ultra-violet intensity of absorption.

If now a selection from both Tables is made of a number of oils practically indistinguishable as regards ultra-violet absorption, it will be seen how very far from real constancy the blue colour intensities can be.

The conclusion is inevitable that either the over-all experimental error of the physical assay is 250 %, or the 606 $m\mu$ chromogen and the substance

Table II. *Cod-liver oils in which the value of* $E^{1\%}_{1\,cm.}$ *328*mμ *is greater than the value of* $E^{1\%}_{1\,cm.}$ *606*mμ.

328 *mμ*	606 *mμ*	328 *mμ*	606 *mμ*
0·14	0·05	1·15	1·0
0·50	0·50	1·15	0·72
0·57	0·50	1·22	1·0
0·58	0·58	1·35	1·25
0·62	0·55	1·37	1·22
0·75	0·57	1·38	1·25
0·75	0·45	1·8	1·78
0·85	0·81	1·86	1·53
0·85	0·66	1·89	1·62
1·07	0·72		

Table III. *Cod-liver oils closely alike as regards ultra-violet absorption but differing in blue value.*

$E^{1\%}_{1\,cm.}$ 328 *mμ*	$E^{1\%}_{1\,cm.}$ 606 *mμ*	$E^{1\%}_{1\,cm.}$ 328 *mμ*	$E^{1\%}_{1\,cm.}$ 606 *mμ*
1·07	0·72	1·15	0·72
1·1	1·18	1·15	1·00
1·05	1·75	1·15	1·47
1·05	1·70	1·15	1·75
1·1	1·68		

responsible for the 328 *mμ* maximum are not simply related. The discrepancy is vastly greater than any experimental error of which we are aware in the physical measurements.

It must however be emphasised that these data embody the results of a very large number of tests on materials ranging from the purest medicinal oil to very crude fish-liver oils of the cattle feeding variety.

A careful inspection of our data on concentrates reveals a similar state of affairs.

Table IV. *To illustrate the more rapid increase in the 620*mμ *band than in the 328*mμ *as richer and richer concentrates are compared.*

$E^{1\%}_{1\,cm.}$ 328 *mμ*	$E^{1\%}_{1\,cm.}$ 620 *mμ*	$E^{1\%}_{1\,cm.}$ 328 *mμ*	$E^{1\%}_{1\,cm.}$ 620 *mμ*
13	22·4	267	650
22	33	275	400
85	174	357	772
112	274	370	950
113	200	500	1140
115	213		

Judging from the mass of data it is impossible to regard these two sets of figures on the blue colour and the ultra-violet absorption respectively as measuring the concentration of the same substance.

We may now consider the data for oils and concentrates in which the intensities of absorption in the colour test have been measured both at 572 and 606 *mμ* (583 and 620 *mμ* for concentrates).

From Table V it will be seen that there is a much closer parallelism between the intensities at 572 and 328 *mμ* than is to be found between those of the 606

Table V.

Material	Ultra-violet absorption		Colour test	
	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ min.	$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$
Codling-liver oil (*a*)	0·125	0·11	0·12	0·06
,, (*b*)	0·22	0·20	0·20	0·13
Cod-liver oil 1	0·38	0·38	0·33	0·33
,, 2	0·55	0·45	0·54	0·54
,, 3	0·55	0·52	0·54	0·62
,, 4	0·55	0·55	0·37	0·47
,, 5	0·57	0·50	0·50	0·50
,, 6	0·57	0·50	0·50	0·58
,, 7	0·67	0·57	0·50	0·61
,, 8	0·75	0·65	0·57	0·57
,, 9	0·82	—	0·83	0·25
,, 10	0·80	—	0·70	0·92
,, 11	0·85	0·55	0·68	0·85
,, 12	0·85	—	0·70	0·88
,, 13	0·95	—	0·92	1·14
,, 14	0·95	0·75	0·81	1·20
,, 15	1·03	0·80	1·02	1·35
,, 16	1·1	—	1·0	1·18
,, 17	1·15	0·95	1·25	1·35
,, 18	1·27	1·05	1·41	1·45
,, 19	1·30	—	1·28	1·50
,, 20	1·37	0·9	1·40	1·22
,, 21	1·40	—	1·37	1·25
,, 22	1·50	0·7	1·65	1·51
,, 23	1·60	—	1·20	1·65
,, 24	1·70	—	1·85	1·90
,, 25	1·65	0·55	1·45	1·92
,, 26	1·80	—	1·43	1·78
,, 27	1·86	1·60	1·74	1·53
,, 28	1·87	—	2·32	1·98
,, 29	1·89	0·50	1·86	1·62
,, 30	1·95	1·0	1·80	2·05
,, 31	4·3	—	3·81	4·76
Fish-liver oil 1	6·2	2·5	5·83	8·91
,, 2	3·67	2·0	3·4	5·1
Halibut-liver oil	11·5	4·0	23	28
Liver oil	4·83	—	4·4	8·5
Concentrate	57·5	—	59·4	101·2
,,	267·0	170	286	650

and 328 $m\mu$ bands. This fact is fully borne out in Table VI, which is merely a representative selection of the data which have been accumulated.

Table VI. *The trend of spectroscopic data with increasing concentration of vitamin A.*

	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ 572 to 583 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 to 630 $m\mu$
Cod-liver oils	1·0	1·0	1·35
Rich fish-liver oils	6·25	5·8	9·0
Concentrates 1	55	60	100
,, 2	267	286	650
,, 3	370	400–500	950

The results of a comparison of biological assays made in London with similar spectroscopic data, support the view which is emerging from this work, namely that the 572 $m\mu$ (583 $m\mu$) chromogen is identical with the substance

responsible for the 328 $m\mu$ band and is in fact vitamin A, whereas the 606 $m\mu$ (620 $m\mu$) band has been recorded at from $\frac{1}{3}$ to 3 times the intensity of the 328 $m\mu$ band, a total variation of the order 10:1, whilst the gross variation for the comparison of the 572 $m\mu$ (583 $m\mu$) intensity at the 328 $m\mu$ band is less than 2:1.

The use of the 328 $m\mu$ band as a quantitative criterion of vitamin A involves the measurement of the gross absorption at 328 $m\mu$, a magnitude theoretically including:

(i) the true contribution of the 328 $m\mu$ band, *plus*

(ii) the contribution of the materials always present in cod-liver oils *etc.*, *i.e.* the normal fatty glycerides and the cholesterol, *plus*

(iii) the contribution of the variable and occasional non-vitamin A constituents, *e.g.* free acids and other impurities.

We have hitherto [Morton and Heilbron, 1928; Drummond and Morton, 1929] used a correction term of 0·13 to 0·22 (log I_0/I for a 1 % solution and a 0·1 cm. cell) for the joint effect of (ii) and (iii) in average medicinal cod-liver oils. Further experience on a wider range of materials has convinced us that the absorption of the fatty glycerides and the cholesterol is negligibly small, and that the excessively high values for the absorption at 328 $m\mu$ arise from the presence of impurities which vary from sample to sample. The correction of the 328 $m\mu$ intensity has therefore been abandoned as too uncertain in its operation. On the whole there is very striking agreement, considering the adverse conditions, between the intensity of absorption at 328 and 572 $m\mu$ (583 $m\mu$). This implies that the molecular extinction coefficient of the 328 $m\mu$ absorption band found in alcoholic solutions is approximately the same as that of the blue pigment band at 572 $m\mu$ resulting from the interaction of antimony trichloride with the chromogen. It is not uncommon in the study of the absorption spectra of related organic compounds to meet cases of bodily wave-length displacement of an absorption band without great change in extinction coefficients as we pass from one substance to others. That such a result is found with liver oils perhaps gives independent support to the view that the ultra-violet band and the 572 $m\mu$ band in the colour test are properties of vitamin A and a derivative respectively.

It may be stated that we have carefully explored the possibility that the intensity of absorption at 328 $m\mu$ is a summation of two factors, one the absorption arising from the 572 $m\mu$ chromogen and the other that due to the 606 $m\mu$ chromogen. This enquiry indicates that the substance responsible for the 606 $m\mu$ band with antimony trichloride is very transparent to light of the wave-lengths near 320–330 $m\mu$.

The views we have advanced necessitate some caution in using the Lovibond technique as a quantitative method of assay. The blue colour is complex, arising from at least two apparently independent absorption bands; in oils we find with antimony trichloride a band in the yellow-green (572 $m\mu$), and a band in the orange (606 $m\mu$), whilst with concentrates the bands are in the

yellow (583 $m\mu$) and the orange-red (620 $m\mu$). Complementary to these bands we have colours which may be roughly described as ranging from violet-blue to greenish-blue. Matching such colours against glasses coloured blue with cobalt gives only a very approximate evaluation of intensities because the procedure rests upon the subjective matching of physically distinct phenomena. Moreover the two absorption bands both count in the matching, so that the colorimetric procedure results in a rough evaluation of the oils as regards the combined quantities of two chromogens present. In actual practice the Lovibond technique gives better results than would be antipicated from this argument, owing to the fact that in a fair percentage of cod-liver oils the relative intensities of the 606 $m\mu$ and 572 $m\mu$ absorption bands are near 1·35:1. A prolonged search has however, as we have shown, made clear that this ratio is by no means to be relied upon in all cases.

We have further obtained definite evidence [Heilbron, Gillam and Morton, 1931] that the concentration of the 606 $m\mu$ chromogen can increase spontaneously without any accompanying change in the 572 $m\mu$ chromogen or the 328 $m\mu$ absorption intensity. Finally we have been able to achieve this increase in the laboratory, so that the existence of two independent chromogens in liver oils seems to be established.

Conclusions.

Liver oils contain two chromogens which, with antimony trichloride, give coloured substances with absorption maxima at 606 $m\mu$ and 572 $m\mu$ respectively. In concentrates these maxima are displaced to 620 $m\mu$ and 583 $m\mu$. A comparison of ultra-violet absorption spectra with spectroscopic data on the colour test discloses (i) that the parallelism between the intensity of the 606 $m\mu$ band and the intensity of the 328 $m\mu$ band breaks down so seriously in extreme cases as to render it improbable that the 606 $m\mu$ chromogen is vitamin A; (ii) that the 572 $m\mu$ chromogen and the substance responsible for the 328 $m\mu$ band are probably identical, (iii) that the blue colour for rich oils and concentrates is often much deeper than would be expected on the basis of correlation between blue colour and ultra-violet absorption. Hence it is concluded that the matching of blue colours with Lovibond glasses, though it may act as a rough guide to vitamin A potency is theoretically unsound.

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CXLVIII. SPECIFICITY IN TESTS FOR VITAMIN A. A NEW CONCEPTION OF THE CHROMOGENIC CONSTITUENTS OF FRESH AND AGED LIVER OILS.

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Evidence is accumulating to the effect that the vitamin A of liver oils is a colourless, or almost colourless substance, characterised by a broad continuous band of selective absorption in the ultra-violet, with a maximum near 328 $m\mu$. The vitamin acts as a chromogen in the sense that with antimony trichloride it gives rise to one and possibly more than one absorption band in the visible.

In the earlier stages of the reaction, the colour arises to a considerable extent from a pronounced absorption band in the yellow-green, the maximum being near 583 $m\mu$ but suffering a variable displacement towards 565–575 $m\mu$ in the presence of certain little-understood, unsaturated substances present in liver oils. The evidence indicates that associated with vitamin A is a substance, which, unlike the vitamin, is highly transparent to light of wave-lengths near 328 $m\mu$ but shares with it the property of acting as a chromogen towards antimony trichloride, giving rise in this case to a coloured compound absorbing maximally in the orange at 620 $m\mu$, the band again undergoing a displacement (to 600–610 $m\mu$) in the presence of certain unsaturated substances of unknown constitution normally present in fish-liver oils.

The two chromogens at present appear to be distinct and separate entities, but their occurrence together, and in quantities not widely different as regards order of magnitude makes it necessary to investigate the possibility of kinship in origin or function.

It is thus clear that the colour test with antimony trichloride occupies a position of cardinal importance in the present phase of development in our knowledge of vitamin A. It is therefore worth while to note the characteristics of the ideal colour test so that the antimony trichloride test may be seen in proper perspective.

The simplest case, in which subjective or objective colorimetry reaches its highest accuracy, is that of solutions containing a single absorbing solute which rigidly conforms with the Lambert-Beer absorption law[1]. The solutions

[1] In any two solutions exhibiting identical absorption of light $c_1 = c_2 d_2/d_1$, where c_1, c_2 are concentrations and d_1, d_2 are thicknesses.

to be compared should not differ in respect of the proportion of any colourless constituents able to depress or enhance the visible absorption of the coloured solute.

The two solutions under comparison should thus exhibit identical spectral absorption curves when the thicknesses are so adjusted that the number of molecules absorbing visible rays is the same in both light paths. Under these circumstances the matching of both solutions separately against coloured glasses is not open to serious objection.

Some of the characteristics of the antimony trichloride colour test, especially as applied to sources of vitamin A may now be noted.

(i) Visible colorations varying from red to blue are given with a wide variety of substances ranging from carotenoids to sterols, and including besides vitamin A, highly unsaturated polyene acids such as the crocetins. There is therefore, in a fundamental sense, nothing very specific about the colour test.

(ii) The reagent itself is a source of difficulty. Ordinary B.P. chloroform contains alcohol, which tends to inhibit the production of the maximum intensity of colour. Specially purified chloroform[1] dissolves appreciably less anhydrous antimony trichloride than B.P. chloroform, and relatively small changes in concentration of antimony trichloride appreciably affect the absolute intensity of the blue colour. The reagent as sometimes used is unstable towards light and moisture, and if kept for a few weeks, even in dark brown bottles, tends to deposit a heavy oily liquid.

(iii) As a criterion of vitamin A, the colour test is applied to a wide variety of substances, including mammalian-liver fats, butter extracts, crude and refined oils from fish livers of many different species, and non-saponifiable extracts at various stages of concentration and diluted in various ways. The "environment" of vitamin A may therefore include sterols, carotenoids and many different saturated and unsaturated substances. It is rarely possible to feel confident that the colour test is free from interference arising either as a result of the presence of chromogens other than vitamin A or as a result of some degree of inhibition. Even restricting attention to one source such as "cod-liver oil," the variations in ordinary physical and chemical properties between individual samples are often very great, quite apart from variations in vitamin A potency. The conditions are so adverse in the sense of a lack of constancy in the environment of vitamin A that the only surprise which need be felt is that the colour test responds as well as it is in fact found to do.

(iv) The colour is transient; not only does it fade, but the rate of fading, the succession of colour changes and the more or less final colour are all subject to considerable variations. There is no doubt that, as at present available (*e.g.* in concentrates), vitamin A itself does not form a stable compound with antimony trichloride and that a succession of secondary actions occurs. Thus the two chromogens which at present appear to be of greatest interest

[1] *Cf.* Report of Cod-Liver Oil Colour Test Sub-Committee, March, 1931, Pharmacopoeia Commission, General Medical Council.

give coloured substances with initial maxima varying from 565 to 585 $m\mu$ and 600 to 624 $m\mu$ depending on the environment. Maxima at 535, 495 and 465 $m\mu$ have all been recorded as the solutions faded. Evidence of other chromogens is provided by the appearance in certain cases of bands at 635, 645, 656, 680 and 690 $m\mu$. All these maxima have been found in fish-liver oils and concentrates. The chromogenic properties of substances in the carotenoid and sterol groups are equally complicated.

(v) The colour test is very sensitive to mass action. In most cases the use of half-strength reagent effects an enormous reduction in the intensity of the blue colour. Even this is not a simple case of mass action, for as we shall show later, it is evident from the spectroscopic data that different chromogens are affected differently by a change in concentration of the reagent.

(vi) It has recently been shown (*cf.* preceding papers) not only that there are two chief chromogens in liver oils, but that the relative proportions may sometimes differ so widely as to effect a gross qualitative change in the shape of the spectral absorption curves characterising the solutions. Thus the 572 $m\mu$ maximum is sometimes vastly stronger than the 606 $m\mu$ maximum and *vice versa*.

In view of the complexity of the phenomena associated with the colour test, the experimental methods must be as searching as possible. Colorimetry, using standard glasses, is of distinct value as a simple and convenient way of expressing colour intensities with some approach to accuracy. For research purposes colorimetry is however of limited value unless frequent parallel spectroscopic determinations are carried out.

If the spectral absorption curves for the blue solutions were always the same, it is indisputable that objective colorimetry using photoelectric cells and recording instruments would provide a highly accurate method for measuring intensities, especially if used in conjunction with a monochromator or a filter capable of eliminating all but a narrow strip of the spectrum. Photoelectric spectrophotometry is however less useful than visual methods for examining the spectra of the blue solutions with which we are concerned, because of the need for rapid detection of variable maxima in the absorption curves. With the Hilger-Nutting spectrophotometer a blue solution can be seen at once to be either normal or abnormal, the wave-length of maximal absorption can be selected, and the intensity of absorption determined, all in a few seconds. The photoelectric technique is unsuitable for exploring spectra, its usefulness is for measuring the intensity of absorption at a given wave-length quantitatively. Unfortunately, each blue solution must be examined visually before the wave-length suitable for the photoelectric method can be selected.

Technique. The following is a description of the technique used in these laboratories.

The reagent (a saturated solution of antimony trichloride in purified chloroform) is kept in the apparatus shown in Fig. 1. The oil or concentrate

is dissolved in pure chloroform, the concentrations chosen being 20 % for an average cod-liver oil and 0·2 % for a concentrate about 100 times as rich as an oil. The solution is placed in the modified pipette shown in Fig. 2, so that

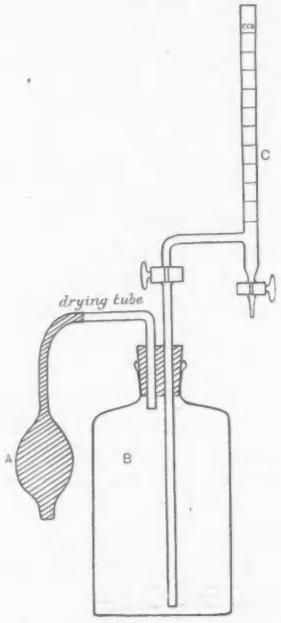


Fig. 1.

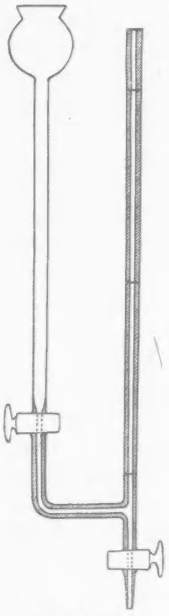


Fig. 2.

Fig. 3.

Fig. 1. Type of apparatus used for storage of antimony chloride in chloroform.
A. Rubber bulb with valve. B. Stock bottle of solution. C. 10 cc. burette.
A drying-tube can be placed between the bulb and the air inlet if the reagent is to be kept in this apparatus.

Fig. 2. Form of pipette used for measurement of chloroform solutions of cod-liver oil (2 cc. graduated in tenths).

Fig. 3. Type of fused quartz cell used for spectroscopic examination of antimony chloride-vitamin A blue colours.

frequent pipetting of chloroform is avoided. We are indebted to Mr H. Rogerson for these simple and efficient methods.

The volume of solution used is 0·3 to 0·6 cc., generally 0·5 cc. This is run into a test-tube, and 5 cc. of antimony trichloride are added. The mixture is at once poured into a 2 cm. fused quartz cell with plane parallel fused-on end plates (*cf.* Fig. 3) which is already mounted in position in the Hilger-Nutting spectrometer. The positions of absorption maxima are first ascertained. The cell is then emptied and the attachment at the spectrometer eye-piece adjusted so that a narrow strip of the spectrum on either side of the maximum is isolated. A fresh mixture is then prepared and the intensity of absorption at the maximum is determined. A number of readings are always made on

fresh mixtures, usually by two observers, and the average values reduced to a basis of 1 %, 1 cm. The ultra-violet absorption spectra are determined in the ordinary way, the results being expressed in terms of log I_0/I (E) at 328 $m\mu$ for a 1 % solution and a 1 cm. cell, no corrections being used unless the persistence of the band is abnormally small, in which case the fact of correction is noted. The numerical values of $E^{1\%}_{1\,\text{cm.}}$ 572 and $E^{1\%}_{1\,\text{cm.}}$ 328 $m\mu$ are in most cases identical within experimental error. We have therefore adopted $E^{1\%}_{1\,\text{cm.}}$ 1·0 as a spectroscopic unit. This value is on the average equal to a blue value (Carr-Price) of 12·5 Lovibond.

It has been shown [Morton, Heilbron and Thompson, 1931] that the blue solutions with liver oils or concentrates may exhibit very marked qualitative differences in absorption spectra, the maximum being liable to occur at 572 $m\mu$ or 606 $m\mu$. Gillam and Morton [1931] on the basis of the comparison of ultra-violet absorption and spectroscopic data on the colour test have shown that an almost exact linear relationship obtains between the intensity of the 328 $m\mu$ maximum and the 572 $m\mu$ chromogen, whereas the relationship between the 328 $m\mu$ band and the 606 $m\mu$ band exhibits a sufficient number of serious discrepancies from linearity to impugn the hypothesis of a common origin. It has also been shown [Coward, Dyer, Morton and Gaddum, 1931], on the basis of the latest and fullest comparison of biological assays with physico-chemical data, that the relative intensities of the 328 $m\mu$ and 572 $m\mu$ maxima provide trustworthy criteria of potency, whereas the values based on the 606 $m\mu$ maximum and on the Lovibond tintometer readings exhibit many anomalies. Further, Lovern and Morton [1931] in studying the oil from the livers of the monk or angler fish observed instances in which the concentration of the 606 $m\mu$ chromogen relatively to the 572 $m\mu$ chromogen was abnormally low, but in which the intensity of the 328 $m\mu$ ultra-violet band corresponded very closely with the intensity of absorption at 572 $m\mu$. The same authors also encountered cases of codling- and cod-liver oils in which similarly low concentrations of the 606 $m\mu$ chromogen were found, although the 572 $m\mu$ chromogen occurred strongly and in amount agreeing with the intensity of the ultra-violet band. We now desire to submit fresh evidence pointing still more definitely to the existence of two distinct chromogens in liver oils.

The claims made by Mittelmann [*cf.* Lovern, Creed and Morton, 1931] seemed to be of special interest as soon as misgivings arose as to the validity of a strict correlation between the unresolved blue colour test and the ultra-violet selective absorption. It became evident in testing the Mittelmann process that the particular oils studied showed an increased chromogen content on standing. The most natural hypothesis to advance as an explanation of such a spontaneous change was that of oxidation. It seemed therefore desirable to ascertain whether the process could be accelerated. Accordingly the following methods were tried.

(i) A current of ozonised oxygen was passed through the oil in the cold for a short time.

(ii) The oil was shaken with about one-fifth its volume of a concentrated solution of hydrogen peroxide for 10–15 minutes, the excess of peroxide washed away with water and the oil dried with anhydrous sodium sulphate and filtered.

(iii) The oil was dissolved in chloroform, a solution of benzoyl peroxide added and the mixture left to stand in the cold for 10–15 minutes. A small quantity of this solution was then used for the antimony trichloride colour test.

(iv) The oil was treated with barium peroxide.

(v) The oil was treated with sodium peroxide.

Method (iv) proved useless and method (v) ineffective in the absence of water, but very striking results were obtained with the other three reagents. The oils investigated were remarkable for the abnormally high $572\,m\mu/606\,m\mu$ intensity ratio, absorption at the latter point being quite feeble. As will be seen from Table I, the treatment with ozone results in a very large increase in the measurable concentration of the $606\,m\mu$ chromogen, the $572\,m\mu$ chromogen and the ultra-violet absorption being but little changed (*cf.* Fig. 4).

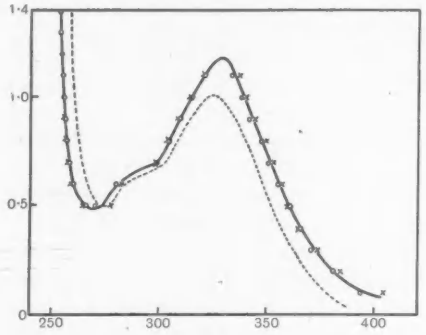


Fig. 4.

—o—o— Steamed C.L.O. 1·5 % (1 cm.).
×—×—× Steamed C.L.O. ozonised 1·5 % (1 cm.).
- - - - - - - Mittelmannised oil from 10 tins 1·5 % (1 cm.).

The enhanced concentration of the $606\,m\mu$ chromogen is well shown in the case of the monk-liver oil. This oil had been obtained a very short time previously by ether-extraction of the livers and had been stored in a glass bottle under ordinary laboratory conditions. The codling oils were obtained by the Mittelmann process, the fresh livers having been sealed in evacuated tins and autoclaved at 120° for 2 hours. The oils were examined within 2–3 days of the fish being caught. Here again the $572\,m\mu$ chromogen predominated to a marked extent over the $606\,m\mu$ chromogen, but the ultra-violet intensity was in good agreement with the higher value. Ozonisation or treatment with peroxides resulted in a very considerable increase in the $606\,m\mu$ chromogen. These experiments were repeated, fresh tins being opened for the purpose. The results proved to be reproducible (*cf.* Table I).

Table I. *The action of O_3, H_2O_2,* etc., *on very fresh liver oils.*

Oil	Remarks	Ultra-violet test $E^{1\%}_{1\,cm.}$ 328 $m\mu$	$SbCl_3$ colour test $E^{1\%}_{1\,cm.}$ 572 $m\mu$	$SbCl_3$ colour test $E^{1\%}_{1\,cm.}$ 606 $m\mu$
Monk-liver oil (pigmented)	As received	0·82	0·825	0·25
	Treated ozone	0·95	0·69	0·96
	Treated H_2O_2	—	0·82	0·96
	+ benzoyl peroxide	—	0·83	1·00
Codling-liver oil (small livers)	As received (liver in tins)	0·12	0·12	0·06
	Same. Next day	0·16	0·12	0·176
Codling-liver oil (larger livers)	As received	0·22	0·21	0·13
	+ O_3. 5 minutes	—	0·19	0·32
	+ O_3. 10 ,,	—	0·215	0·29
	+ O_3. 20 ,,	—	0·18	0·31
	+ O_2. 20 ,,	—	0·19	0·13
Codling-liver oil (larger livers)	As received	0·19	0·18	0·125
	+ O_3. 1 minute	—	0·17	0·14
	+ O_3. 3 minutes	—	0·175	0·20
	+ O_3. 5 ,,	—	0·175	0·25
	+ O_3. 10 ,,	0·19	0·17	0·27
	Treated H_2O_2	0·21	0·175	0·32
Cod-liver oil	As received	0·73	0·76	0·355
	+ O_3. 20 minutes	0·73	0·59	0·93
Cod-liver oil	As received	0·69	0·72	0·39
	+ O_3. 30 minutes	0·73	0·72	0·93
	After 11 days	0·75	0·75	1·00

Later tests showed that the unusual preponderance of the 572 $m\mu$ chromogen was not due to the Mittelmann process *per se* (*cf.* Lovern, Creed and Morton, 1931), since, using the same livers, the 572 $m\mu$ chromogen predominated in the fresh steamed oil and in the oil from newly opened tins. It is significant however that a threefold increase in the 606 $m\mu$ chromogen was obtained on treatment with ozone, hydrogen peroxide or benzoyl peroxide.

On examining our records for cod-liver oils which when first tested had shown the 572 $m\mu$ chromogen preponderating over the 606 $m\mu$ chromogen, a number of these were discovered, and all were re-examined for ultra-violet absorption and by the antimony trichloride colour test. It was found that in each of these, after the oil had stood for several months, a significant change had occurred, the concentration of the 606 $m\mu$ chromogen having increased considerably. The ultra-violet absorption exhibited a very much smaller increase in intensity, whilst the 572 $m\mu$ band was also but little changed. Each oil was then subjected to treatment with ozone or hydrogen peroxide, and in some cases it was found that the "ageing" process could be carried still further. In other cases it was found that the ageing process had reached a maximum, and that further treatment with ozone or hydrogen peroxide merely resulted in the gradual destruction of both chromogens as evidenced by lessened absorption both at 572 and 606 $m\mu$ in the colour test and at 328 $m\mu$ in the ultra-violet absorption (Table II).

We have thus demonstrated for eight cod-liver oils, two codling-liver oils and at least one monk-liver oil that two chromogens can be detected in the oils. Spontaneous or induced changes, which may be oxidations, result in a

Table II. *Spectroscopic data on the spontaneous and the accelerated ageing of cod-liver oils.*

		Ultra-violet test		Colour test	
Oil	Remarks	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ min.*	$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$
1	As received	1·37	0·90	1·40	1·22
	2 months later	1·42	0·95	1·33	1·20
	Treated O_2	1·40	—	1·41	2·12
	Treated H_2O_2	—	—	1·52	2·42
2	As received	1·86	1·66	1·74	1·53
	5 months later	2·0	1·73	1·90	2·55
	Treated H_2O_2	—	—	1·90	2·83
3	As received	1·87	1·26	2·32	1·98
	7 months later	2·25	1·53	2·01	2·75
	Treated H_2O_2 (overnight)	—	—	1·75	2·50
4	As received	1·90	0·67	1·86	1·62
	5 months later	2·03	0·80	1·95	2·33
	Treated H_2O_2	—	—	2·09	2·70
5	As received	1·52	0·72	1·62	1·51
	5 months later	1·60	0·95	1·52	2·02
	Treated H_2O_2	—	—	1·41	2·05
6	As received	1·07	—	1·07	0·72
	7 months later	1·17	—	1·14	1·75
7	As received	1·23	0·90	1·35	1·52
	Treated H_2O_2	—	—	1·32	1·90

* This is the intensity of absorption at the minimum but the wave-length is variable.

very considerable increase in one chromogen before any apparent change occurs in the quantity of the other chromogen. On the other hand, the ultra-violet absorption is not appreciably affected by the spontaneous or induced enhancement of the 606 $m\mu$ chromogenic value. It is however undesirable to generalise from these results, for we have examined the question whether all absolutely fresh oils contain less of the 606 $m\mu$ chromogen than of the 572 $m\mu$ chromogen, but by some spontaneous or induced change can be enriched in the 606 $m\mu$ chromogen. Definite evidence has been obtained to the effect that in a number of quite fresh oils the 606 $m\mu$ band not only predominates slightly over the 572 $m\mu$, but is present at its full intensity, ozonisation effecting no increase.

In our experience, an oil in which the 572 $m\mu$ chromogen predominates over the 606 $m\mu$ chromogen is somewhat unusual. The two chromogens are so far not demonstrably related and there is thus no reason to assume a constant ratio either for the 572 and the 606 $m\mu$ intensities or the 572 and the potential 606 $m\mu$ intensity. The lack of constancy in the ratios (Table III) further emphasises the independence of the two chromogens, although the differences are not so great as to preclude kinship.

In evaluating the characteristics of concentrates we have repeatedly noticed that the "blue value" increases much more rapidly than the intensity of absorption in the ultra-violet. This is probably analogous to an observation made by Smith and Hazley [1930].

Table III.

$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$ (maximum attainable)	Ratio 606 : 572 $m\mu$
1·4	2·4	1·71 : 1
1·75	2·85	1·63 : 1
2·3	2·5	1·09 : 1
1·86	2·7	1·45 : 1
1·62	2·05	1·27 : 1
1·07	1·75	1·64 : 1
1·35	1·90	1·41 : 1
0·82	1·0	1·22 : 1
0·21	0·32	1·52 : 1
0·18	0·32	1·77 : 1
0·76	0·93	1·22 : 1
0·72	1·0	1·37 : 1

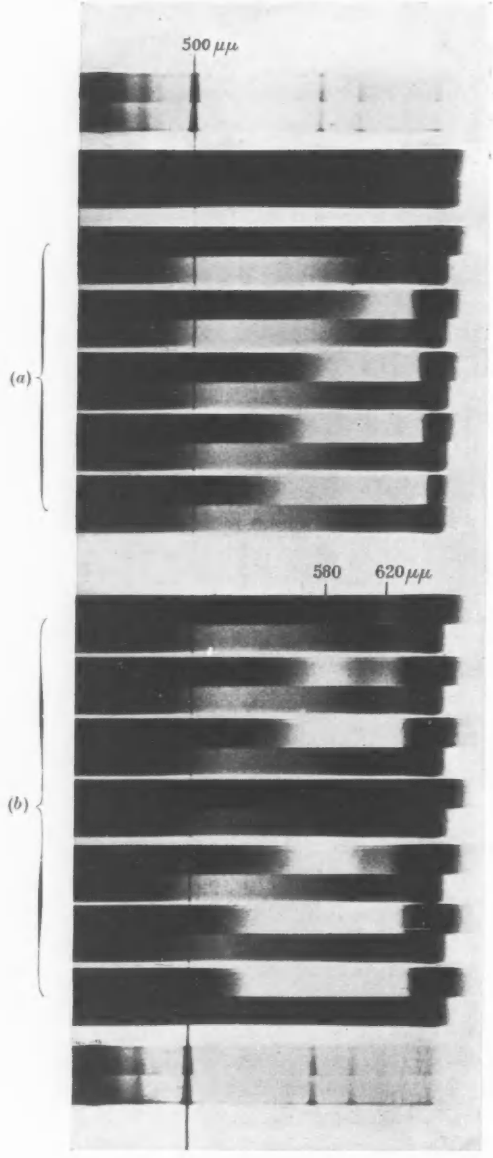
During the process of saponification the 572 $m\mu$ chromogen or its environment is changed so as to displace the maximum to 583 $m\mu$, but for this chromogen the increase in the intensity of absorption given by the colour test is exactly proportional to the degree of concentration effected. On the other hand, if the oil is such that the 606 $m\mu$ band fails to reach the maximum intensity possible for the oil, then for this second chromogen the increase in absorption surpasses that to be expected from the degree of concentration. In addition, a displacement to 620 $m\mu$ occurs. When concentrates are dissolved in either seal oil or whale oil the two revert to 606 and 572 $m\mu$, but in arachis oil no such reversion takes place. Clearly therefore the displacement of the maxima is somehow connected with the nature of the medium.

The results with concentrates are of importance in connection with the views of Norris and Church [1930, 1, 2]; *cf.* Smith and Hazley [1930]. In carrying out quantitative studies concerning the validity of the colorimetric test these authors were led to conclude that liver oils contain a saponifiable substance which tends to inhibit the production of the blue colour. Norris and Church [1930, 2] found that addition of oleic acid to concentrates produced the same inhibitory effect.

Separation of the two absorption maxima (colour test).

Spectroscopic examination of the blue solution obtained by the interaction of the antimony trichloride reagent and the majority of liver oils and concentrates shows the absorption curve to be unsymmetrical, with the sharper decrease of absorption occurring on the long-wave side of the maximum, Fig. 5 (*b*). With the anomalous oils, however, the curve is still unsymmetrical but in the opposite sense, Fig. 5 (*a*), the steep descent being now on the side of shorter wave-lengths. Now in most cases, starting from an oil giving higher absorption at 572 than at 606 $m\mu$, ozonisation or treatment with hydrogen peroxide effects so great an increase in the absorption at 606 $m\mu$ that the curve becomes of the type shown in Fig. 5 (*b*).

As we have seen, the interpretation advanced in this paper involves the view that an inflexion indicates the presence of a partially masked absorption



Aluminium spark.

Tungsten spark under water (control).

Cell	log I_0/I	Halibut oil cc.	$SbCl_3$ reagent cc.
2 cm.	0·8	0·05	5
,,	,,	0·10	5
,,	,,	0·15	5
,,	,,	0·20	5
,,	,,	0·25	5

Cell = 2 cm. log I_0/I = 0·8.

Halibut oil cc.	$SbCl_3$ reagent cc.	Interval min.	$SbCl_3$ reagent cc.
0·10	0·3	1	4·7
0·15	0·3	1	4·7
0·20	0·3	1	4·7
0·10	0·3	2	4·7
0·20	0·3	2	4·7
0·30	0·3	2	4·7
0·40	0·3	2	4·7

Aluminium spark.

(*a*) illustrates the appearance of one band in the colour test;
(*b*) ,, ,, ,, two bands ,, ,,

band. It would however be an advantage if the real existence of the 572 $m\mu$ band in normal oils could be demonstrated even more clearly. We have found this to be possible by following the procedure described below.

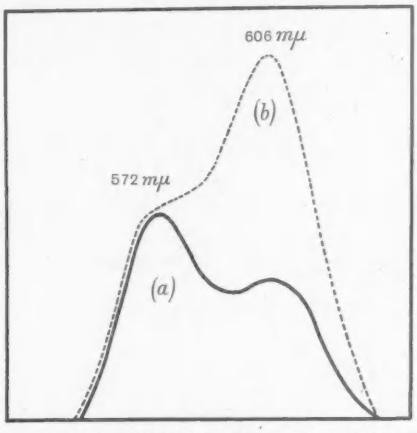


Fig. 5. Diagrammatic representation of the absorption spectra of the blue solutions produced with (*a*) certain fresh oils, (*b*) the same after spontaneous or induced ageing.

The usual quantity (0·5 cc.) of a solution of liver oil in chloroform is run into a test-tube and a small quantity (0·3 cc.) of the antimony reagent added. The solution is then allowed to stand for 1 minute and the remainder (4·7 cc.) of the reagent is then added and the solution examined spectroscopically. In nearly every case two separate absorption bands with maxima at 565–575 and 600–620 $m\mu$ can be seen very clearly. The intensity of the former band is practically the same as when the whole of the reagent is added at once, but the latter band is very much reduced in intensity.

It has been possible to photograph solutions treated in this way and the results are reproduced in Plate VI, whilst Fig. 6 shows a schematic representation of the absorption curves of the various coloured substances.

Table IV illustrates the results obtained by the alternative procedures.

Table IV.

		$E^{1\%}_{1\,cm.}$ 600–612 $m\mu$	$E^{1\%}_{1\,cm.}$ 572–575 $m\mu$
Halibut oil 1.	Procedure (*a*)*	61·2	35·7
	,, (*b*)	27·3	36·4
Halibut oil 2.	,, (*a*)	101	58·5
	,, (*b*)	62	50
Cod-liver oil 1.	,, (*a*)	1·05	0·9
	,, (*b*)	0·80	0·85
Cod-liver oil 2.	,, (*a*)	2·75	2·2
	,, (*b*)	1·87	2·1
Cod-liver oil 3.	,, (*a*)	0·85	0·72
	,, (*b*)	0·52	0·70

* (*a*) Normal procedure. (*b*) Preliminary addition of a small quantity of $SbCl_3$ reagent.

The striking phenomenon about the procedure involving the preliminary addition of a small quantity of reagent is that the reduction in intensity of

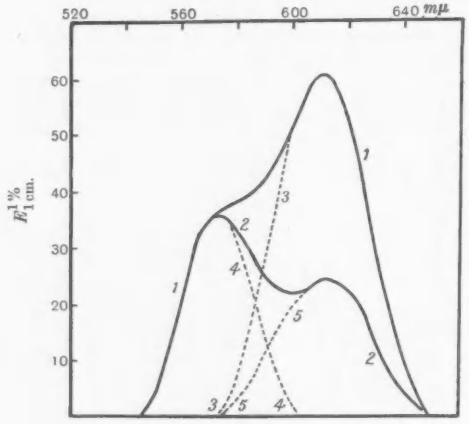


Fig. 6.

1. Schematic representation of absorption curves of the antimony trichloride blue colour with a halibut oil, measured on the freshly mixed solution, the reaction being carried out in the normal way (*a*).
2. Ditto, the reaction being carried out adding a small quantity of $SbCl_3$ reagent 1 minute before adding the bulk of the reagent (*b*).
3. Ditto, the 606 *mμ* band in case (*a*).
4. Ditto, the 572 *mμ* band in cases (*a*) and (*b*).
5. Ditto, the 606 *mμ* band in case (*b*).

1. Summation of 3 and 4. 2. Summation of 4 and 5.

the blue colour is substantially confined to the 606 *mμ* band, while the 572 *mμ* band is obtained at almost its full intensity.

Another but less satisfactory method whereby the two absorption bands can be manifested is to vary the concentration of antimony trichloride in the reagent.

Table V indicates the values obtained with a rich cod-liver oil.

Table V.

	$E^{1\%}_{1\,cm.}$ 606 *mμ*	$E^{1\%}_{1\,cm.}$ 572 *mμ*
Saturated solution of antimony trichloride (in B.P. chloroform previously treated with anhydrous calcium chloride)	5·23	4·05
Two-thirds saturated reagent	3·9	3·6
Half saturated reagent	3·2	3·1

With the full strength reagent the 606 *mμ* maximum is shown very clearly and an inflexion is seen at 572 *mμ*, but with the diluted reagents two bands are visible separately, although neither is present at its full intensity.

The reality of two absorption bands in the coloured solutions is thus established beyond question and the variations in the relative intensities of the

two bands under different conditions go a long way towards accounting for the non-linear colour-concentration curves which have been recorded by many investigators.

Discussion.

Without prejudicing the issue concerning vitamin A, the physical data lead us to postulate the following entities.

(i) A substance X, which gives rise to the ultra-violet absorption with its maximum at $328 m\mu$.

(ii) The chromogenic substance X' in oils which gives rise to the $572 m\mu$ maximum.

(iii) The chromogenic substance X'' present in concentrates giving rise to the $583 m\mu$ maximum.

(iv) The chromogenic substance Y' in oils responsible for the $606 m\mu$ maximum.

(v) The chromogenic substance Y'' of concentrates causing the band at $620 m\mu$ in the colour test.

(vi) A substance giving rise to an absorption band with a maximum near $280 m\mu$.

(vii) The substance or substances which react with antimony trichloride to give coloured compounds with maxima at 640–650 $m\mu$ and 690–700 $m\mu$.

Confining our attention for the present to the first five, the comparison of intensities of absorption at 328 and $572 m\mu$ for oils leads to the view that X and X' are identical. Similar comparisons using concentrates indicate that X and X'' are identical. This leads to the possibility that X' is an ester and X'' the alcohol derived from it on saponification. This suggestion does not however provide an explanation of the variability of the maximum in the colour test, because (*a*) in liver oils (*e.g.* halibut oils) which have much higher blue values than cod-liver oils there is a progressive displacement from 570 to $582 m\mu$, the very rich oils being chromogenically very like concentrates, and (*b*) when a concentrate is dissolved in seal oil or whale oil the maximum for the solution appears at 570, but if arachis oil is used it remains at $583 m\mu$.

Exactly similar considerations lead to the view that the $606 m\mu$ chromogen Y' is identical with the $620 m\mu$ chromogen Y'', the differences being due entirely to the displacing action of unsaturated substances. This does not however account for the apparent increase in the 606–620 $m\mu$ chromogen in certain oils on ozonisation or treatment with hydrogen peroxide. The following three possible explanations of the phenomena suggest themselves, but the task of ascertaining which is the true one promises to be unusually difficult.

(1) Vitamin A, which we may regard as the material responsible for the $328 m\mu$ maximum, is the chromogen responsible for both the 572–583 $m\mu$ band and the 606–620 $m\mu$ maximum. Antimony trichloride is known to form the double compounds:

$$C_6H_4(CH_3)_2, SbCl_3 \qquad C_6H_4(CH_3)_2, 2SbCl_3 \textit{ etc.},$$

so that there is nothing inherently impossible in the compounds $SbCl_3$, vitamin A; $2SbCl_3$, vitamin A.

(2) There are two independent chromogens *X* and *Y* present in the oil. The absolute amount of *Y* present is not increased by ozonisation *etc.*, but the reaction

$$Y + SbCl_3 \rightarrow SbCl_3, Y \text{ (blue substance)}$$

only occurs to its full extent in the absence of certain "inhibitors" which are eliminated by treatment with ozone or hydrogen peroxide.

(3) The quantity of *Y* present in an oil undergoes a real increase on ozonisation by virtue of the presence of a precursor substance, which is converted into *Y* either by spontaneous ageing or accelerated oxidation. Such a precursor, if it exists, must be chromogenically inert, or relatively so, and will absorb rays of wave-lengths near 328 $m\mu$ comparatively feebly.

The explanation given under (1) involves that the "chromogens" are not capable of separation, it accounts at once for the somewhat startling fact that an animal depleted of vitamin A but supplied with carotene accumulates both "chromogens" in the liver, but it demands for the ozonisation experiments the removal of "inhibitor" substances, which before ozonisation can form complexes with antimony trichloride to such an extent as to modify profoundly the equilibria:

$$\text{Vitamin A} + SbCl_3 \rightleftharpoons SbCl_3, \text{ vitamin A.}$$
$$SbCl_3, \text{ vitamin A} + SbCl_3 \rightleftharpoons 2SbCl_3 \text{ vitamin A.}$$

The inhibitor substances must however remove antimony trichloride from the sphere of action of vitamin A without themselves forming coloured substances. The fact that changes in the concentration of the reagent markedly affect the relative intensities of the 572 and 606 $m\mu$ bands is consistent with an explanation attributing a dominant rôle to mass action. The idea that both bands arise from vitamin A is attractive because of its avoidance of the complication of a new chromogen, but its weaknesses are patent in view of the very hypothetical nature of the essential "inhibitors" and the difficulty of reconciling it with the results of a preliminary addition of the antimony trichloride reagent. Finally, it would seem reasonable to expect the full development of the 606 $m\mu$ band to be at the expense of the 572 $m\mu$ band, and this is not realised.

The explanation given under (2) enables us to retain temporarily the validity of the 606 $m\mu$ band as a possible criterion of vitamin A (although probably forcing us to abandon the 328 $m\mu$ band) without postulating a threefold increase in potency on ozonisation. It again however compels us to attribute a very important rôle to the inhibitors about which so little is known. It minimises the possibility of a simple correlation between ultra-violet absorption or feeding tests and the colour value and gives no rational account of the apparent correlation between the 328 and 572 $m\mu$ bands.

Explanation (3) seems on the whole to fit the facts best, but its consequences are serious. It involves the view that tintometric methods of assay can only be regarded as good approximations. It requires two new substances, a chromogen and a chromogen precursor, the absorption spectra of which in the ultra-violet have so far eluded our attempts to discover them. It fits in extremely well with the best biological assays to which we have access, but it raises the question of the function of the new chromogen and its precursor.

From whatever angle the new data are regarded a plurality of working hypotheses seems inevitable. Whilst we have frankly envisaged the difficulties, it is necessary to define our attitude. The most plausible interpretation leads us to suggest the following physico-chemical criteria for vitamin A.

(i) A colourless or pale yellow substance exhibiting selective ultra-violet absorption free from fine structure and absorbing maximally at or near 328 $m\mu$, and (ii) giving with antimony trichloride a coloured substance characterised by a maximum in the yellow between 580 and 590 $m\mu$, whilst the molecular extinction coefficients at 328 and 580–590 $m\mu$ expressed in terms of vitamin concentration should be approximately equal.

These criteria are not free from a measure of uncertainty from the strictly numerical standpoint, because the change in environment involved in the preparation of rich concentrates displaces the chromogen from 572 to 583 $m\mu$, the vitamin itself remaining a minor constituent of even the richest concentrates so far obtained. Further concentration might result in a continuation of this shift in wave-length.

Again, antimony trichloride produces coloured substances with a wide range of sterol and carotenoid derivatives, and in many cases the absorption maxima are found in the yellow-red region of the spectrum. It would be by no means surprising if the 583 $m\mu$ maximum were found to be by itself non-specific for vitamin A. Again, an absorption maximum near 320–330 $m\mu$ in the ultra-violet has never been claimed as a specific test for vitamin A, it merely indicates a property of vitamin A. There would be nothing very unexpected in the discovery of a sterol or carotenoid derivative exhibiting both a maximum near 580-590 $m\mu$ with antimony trichloride and an absorption band very like that observed in liver oils, the substance being still distinct from vitamin A. It would however be much less probable to find the same relative intensities of absorption at 328 and 583 $m\mu$. The nearest approach to satisfying these criteria has been obtained with "dihydrocarotene" prepared by the method of Smith [1931]. This product, which is undoubtedly a mixture, gives selective absorption in the near ultra-violet and with antimony trichloride a blue solution exhibiting maxima at 583 and 648 $m\mu$ [Heilbron and Morton, 1931]. The interpretation of these results must await the examination of the separate constituents present in the mixture, both by biological and physico-chemical methods [*cf.* Drummond and Ahmad, 1931; Karrer, Euler and Hellström, 1931].

SUMMARY.

1. The ideal conditions for accurate colorimetric determinations are considered, and the actual characteristics of antimony trichloride as a colour-producing reagent are reviewed in detail.

2. In a considerable number of oils characterised in the colour test by predominance of the 572 $m\mu$ band over the 606 $m\mu$ band, a large increase in the intensity of the latter band can be obtained by treating the oil beforehand with ozonised oxygen, hydrogen peroxide or benzoyl peroxide.

Oils which initially showed an excess of the 572 $m\mu$ chromogen over the 606 $m\mu$ chromogen undergo a slow spontaneous ageing which results in a marked increase in the intensity of the 606 $m\mu$ band. Increases in the 606 $m\mu$ absorption are not at the expense of the 572 $m\mu$ chromogen and are not accompanied by similar increases in the latter, and the absorption at 328 $m\mu$ remains practically constant throughout.

3. The reality of the two chromogens and the possibility of recording the 572–583 $m\mu$ band even in oils and concentrates in which the 606–620 $m\mu$ chromogen predominates can be demonstrated visually and photographically by a modification of the ordinary procedure in the colour test, which suppresses or eliminates some of the 606 $m\mu$ chromogen.

4. The bearing of the data on the vitamin A problem is discussed in detail, and the most plausible criteria for the vitamin are stated.

5. The two main chromogens behave as if they were separate and distinct entities but a close genetic relationship seems highly probable.

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CXLIX. THE PHYSIOLOGICAL FUNCTION OF VITAMIN B_1.

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(*Received July 2nd, 1931.*)

More striking among the theories which have been proposed to explain the mechanism of action of "vitamin B" (using the old terminology) are those that connect the vitamin with biological oxidation processes. Some investigators have considered the vitamin to be concerned in cellular respiration in general, while others have claimed the existence of a definite relationship between the vitamin and certain aspects of metabolism. Some workers, however, have opposed the evidence on which such claims are based. *Post mortem* examination of avitaminous animals, while affording much valuable information, has not led to any definite knowledge as to the rôle that the vitamin plays in body economy. In some recent experiments with rats we observed that if the vitamin B_1-deficient animals were killed before they passed into the moribund condition, no specific lesions could be found in the internal organs, although the deprivation of vitamin B_1 was quite severe. These observations suggest that the gross abnormalities observed in the extreme stage of vitamin B_1 deficiency are most probably the secondary results of a primary disorder [*cf.* Drummond and Marrian, 1926].

The present investigation represents an attempt to throw some light on this problem.

Method.

All work was carried out with rats, most of which were of the black and white strain. They were kept in separate cages and 2 drops of a potent cod-liver oil were fed daily to each of them apart from the basal diet. All other supplements were also separately administered unless otherwise mentioned.

A. Vitamin B_1 and the protein/carbohydrate ratio of the diet.

The amylum-toxin theory originally proposed by Eijkman [1897, 1, 2], which was opposed by Grijns [1901, 1907] and later discarded, has more than once been revived in modified forms [Funk, 1914; Randoin and Simonnet, 1924]. Randoin and Simonnet, working with pigeons, considered that there was a definite relationship between the "antineuritic" vitamin and the carbohydrate content of the diet, while Hartwell [1925] and Reader and Drummond [1926] found that rats on high-protein diets required larger allowances of

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"vitamin B." It was, however, subsequently shown by Hassan and Drummond [1927] that the corrective agent for high-protein diets present in marmite was a heat-stable substance and, therefore, presumably not vitamin B_1.

In the present experiments the effect of the same optimum and sub-optimum doses of a relatively concentrated preparation of vitamin B_1 on the growth of groups of young rats subsisting on diets of varying carbohydrate/protein ratios was studied. It was considered that if vitamin B_1 plays a specific part in carbohydrate metabolism, sub-optimum doses of vitamin B_1 might have a greater influence on the growth of young rats receiving less carbohydrate than of those receiving more. The deleterious effect of excess protein in the diet, if any, was sought to be corrected by Hassan and Drummond's technique of increasing the quantity of alkaline-autoclaved marmite in the daily ration. In order to keep the conditions comparable, however, the same quantity of alkaline-autoclaved marmite was incorporated in high-protein as well as in low-protein diets. The animals were allowed to eat *ad libitum*.

Two litters containing six piebald rats each were broken up as evenly as possible as regards weight and sex into groups of two.

The following diets were used:

Diet 1	%	Diet 2	%	Diet 3	%
Sucrose	25	Sucrose	50	Sucrose	75
Caseinogen	71	Caseinogen	46	Caseinogen	21
Salt mixture (McCollum)	4	Salt mixture (McCollum)	4	Salt mixture (McCollum)	4

The caseinogen used was the "light, white casein" of the British Drug Houses. 20 cc. of a 50 % solution of alkaline-autoclaved marmite (prepared by autoclaving a concentrated aqueous solution of marmite with baryta at p_H 9 for 1½ hours at 18 lbs. pressure and subsequently removing the baryta by sulphuric acid) were incorporated per 100 g. of the basal diet. The animals received cod-liver oil and the vitamin B_1 preparation separately.

Table I shows the diet and the dose of the vitamin B_1 preparation received by each group of animals.

Table I.

Group	Basal diet	Dose of vitamin B_1 preparation* (cc.)
A	1	0·075
B	2	0·075
C	3	0·075
D	1	0·15
E	2	0·15
F	3	0·15

* Obtained by extracting brewer's top yeast with 50 % aqueous alcohol, concentrating the extract *in vacuo*, precipitating with neutral lead acetate and removing the lead from the filtrate by hydrogen sulphide. The optimum dose of the concentrated solution was 0·15 cc., containing about 30 mg. organic material [see Guha, 1931, 1].

The growth curves, shown in Figs. 1–3, indicate that the need for vitamin B_1 is not diminished with a reduction in the carbohydrate content of the diet.

It will be observed that animals on the high-protein diet exhibit somewhat less vigorous growth than the others, though no definite signs of ill-health

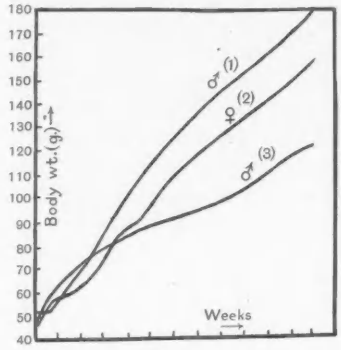

Fig. 1.

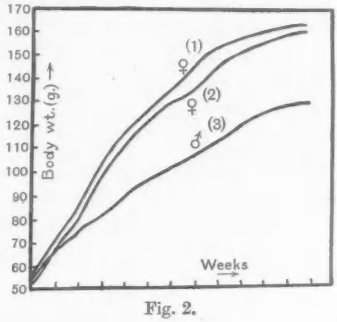

Fig. 2.

Fig. 1. Diet 1. Curves (1) and (2); dose = 0·15 cc. lead filtrate. Curve (3); dose = 0·075 cc. lead filtrate.

Fig. 2. Diet 2. Curves (1) and (2); dose = 0·15 cc. lead filtrate. Curve (3); dose = 0·075 cc. lead filtrate.

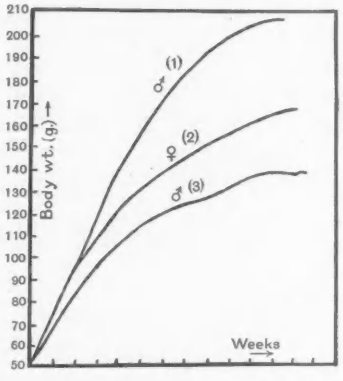

Fig. 3. Diet 3. Curves (1) and (2); dose = 0·15 cc. lead filtrate. Curve (3); dose = 0·075 cc. lead filtrate.

were noticed. The high marmite content of these diets tended to give the rats slight diarrhoea during the first few days of experiment, which, however, rapidly cleared up.

B. The relation between vitamin B_1 and the nature of the dietary carbohydrate.

Considering the question of a possible relationship between vitamin B_1 and carbohydrate metabolism, it was of interest to investigate the effect of

a sub-optimum dose of vitamin B_1 on the growth of young rats receiving diets containing different carbohydrates. It has been observed [Campbell and Soskin, 1928; Campbell and Maltby, 1928; Rose, Giragossintz and Kirstein, 1929–30] that the administration of dihydroxyacetone and fructose by way of the small intestine is followed by a rise in blood-lactic acid, whereas aldoses like dextrose and galactose have no such effect. As Kinnersley and Peters [1929] have stated that avitaminosis B_1 in the pigeon results in the production of excess lactic acid, it was clearly desirable to know whether the requirement of vitamin B_1 would vary according as dextrose or fructose formed the carbohydrate in the diet, although the observations of Campbell and of Rose constitute no evidence that lactic acid is produced in the normal metabolism of ingested fructose.

In these experiments the following basal diets were used.

Table II.

Diet No.	Constituents of diet		
4	75 % dextrose;	21 % caseinogen*;	4 % salt mixture†
5	,, fructose;	,,	,,
6	,, sucrose;	,,	,,
7	,, galactose;	,,	,,
8	,, lactose;	,,	,,
9	,, maltose;	,,	,,

* B.D.H. "light, white casein." † McCollum's.

A litter of 8 young rats was split up into 3 groups, (*a*), (*b*) and (*c*), and fed as shown in Table II. Their growth curves are illustrated in Figs. 4–6, and their average weekly gains in weight are given in Table III.

Table III.

Rat No.	Sex	Group	No. of basal diet	Daily dose of vitamin B_1 (lead filtrate) cc.	Av. weekly gain in weight over 18 weeks g.
1	♂	(*a*)	4	0·075	3·8
2	♀	(*a*)	4	0·075	3·8
3	♀	(*a*)	4	0·075	3·7
4	♂	(*b*)	5	0·075	3·3
5	♀	(*b*)	5	0·075	4·2
6	♀	(*b*)	5	0·075	3·4
7	♀	(*c*)	6	0·075	3·8
8	♀	(*c*)	6	0·075	3·8

The vitamin B_1 administered (0·075 cc.) represents a sub-optimum dose. Each animal received the vitamin B_1 supplement, 1·5 cc. of 50 % alkalised marmite and 2 drops of cod-liver oil daily in a dish separately from the basal diet.

The figures of the growth rates given in Table III show that there is no special relation between the requirement of vitamin B_1 and the presence of dextrose, fructose or sucrose in the diet. When some of these animals were later (after about 25 weeks) deprived of vitamin B_1, those that were receiving fructose were found to decline at a somewhat slower rate than those receiving

dextrose or sucrose. This, however, could not be repeated when Kahlbaum's "crystallised laevulose" was used instead of the B.D.H. product. It is possible

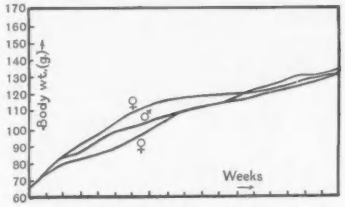

Fig. 4. Diet 4; dose = 0·075 cc. of lead filtrate.

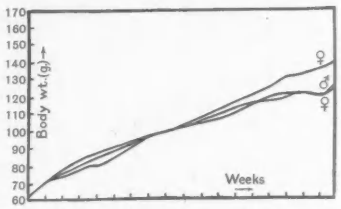

Fig. 5. Diet 5; dose = 0·075 cc. of lead filtrate.

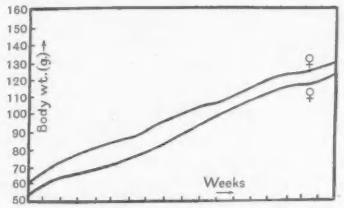

Fig. 6. Diet 6; dose = 0·075 cc. of lead filtrate.

that this latter carried traces of vitamin B_1, although it gave no perceptible precipitate with phosphotungstic acid.

When diets 7 and 8, containing galactose and lactose respectively as the sole sources of carbohydrate, were used, it was found that they caused a rapid decline of both young and adult rats. In the case of diet 7 final collapse could not be prevented even by incorporating whole dried yeast (5 %) in the diet. This phenomenon has been further investigated separately [Guha, 1931, 2].

Diet 9 containing maltose produced as good growth as the diet containing dextrose.

C. The relation between vitamin B_1 and dietary fat.

In the course of investigations on the effect of purified rations on the growth of rats, Evans and Lepkovsky [1929] made the striking observation that the presence of fat in the diet diminished the need of the rat for vitamin B_1. As the importance of this observation in relation to the suggested rôle of vitamin B_1 in carbohydrate metabolism is obvious, this question was investigated with reference to the following points.

(1) The effect of a sub-optimum dose of vitamin B_1 on the growth of young animals receiving diets containing (*a*) varying amounts of the same fat; (*b*) different fats in the same proportion; (*c*) varying proportions of fat but the same restricted amount of food every day.

(2) The effect of a complete deficiency of vitamin B_1 on rats subsisting on diets containing varying amounts of fat.

The following basal diets were used. The protein in each of the diets consisted of "light, white casein" (B.D.H.).

Table IV.

	Diet 12 %	Diet 13 %	Diet 14 %
Sucrose	65	35	10
Palm kernel oil	10	40	65
Caseinogen	20	20	20
Salt mixture (McCollum)	5	5	5

	Diet 24 %	Diet 25 %	Diet 26 %
Sucrose	65	35	10
Olive oil	10	40	65
Caseinogen	20	20	20
Salt mixture	5	5	5

	Diet 28 %	Diet 29 %	Diet 30 %
Sucrose	65	35	10
Lard	10	40	65
Caseinogen	20	20	20
Salt mixture	5	5	5

	Diet 44 Parts		Diet 45 Parts		Diet 49 Parts
Lard	50	Sucrose	50	Sucrose	70
Caseinogen	36	Caseinogen	36	Caseinogen	20
Salt mixture	4	Salt mixture	4	Salt mixture	4

Of these, diets 44, 45 and 49 were incorporated with 20 cc. of 50 % alkalised marmite per 100 g. of the diet. The caseinogen content in diets 44 and 45 was raised in order to bring the nutritive ratio approximately to 1:3 and thus to make them more balanced than the other high fat diets [see Evans and Lepkovsky, 1929]. Diets 44 and 49 were made up so as to resemble Evans and Lepkovsky's diets 559 and 542 respectively. When the other basal diets were used, 1 cc. of alkalised marmite was fed separately every day. This dose was considered sufficient as 2 cc. gave no better growth. Two drops of cod-liver oil were, as usual, fed separately to each rat. Vitamin B_1, whenever fed, was administered separately in a sub-optimum dose (0·05 cc. of a lead filtrate preparation from brewer's yeast, different from that used for work described in the preceding sections).

Litters were evenly broken up as regards weight and sex and litter-mates used for the same batch of experiments. Some animals were kept under observation for periods exceeding 25 weeks. Figs. 7–9 illustrate some of the results obtained. Food intake records, which were kept for a period of 3 months, show that in general the gain in weight goes hand in hand with the food consumption. It will be observed that while olive oil and palm kernel oil do not give any definite indication of a sparing action on vitamin B_1 when the animals are receiving a sub-optimum dose of vitamin B_1, lard appears to have a definite effect. The decline in weight of rats completely deficient in vitamin B_1 is also slower when lard is present in the diet.

Four rats which were receiving diets 28 and 29, containing respectively 10 and 40 % lard, supplemented by a sub-optimum dose of vitamin B_1, were

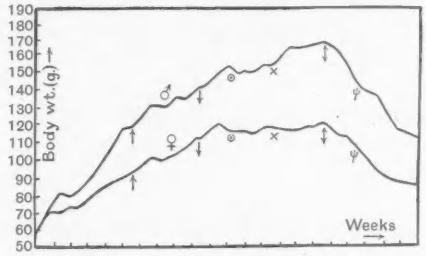

Fig. 7. Dose of vitamin B_1 = 0·075 cc. of lead filtrate throughout except as stated below; basal diet 12 from beginning of experiment; ↑ diet 24; ↓ diet 28; ⊙ restricted food intake; × food *ad libitum*; ↕ diet 45, B_1 supplement stopped; ψ diet 49 (no B_1).

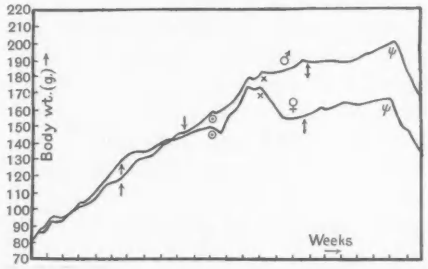

Fig. 8. Dose of vitamin B_1 = 0·075 cc. of lead filtrate throughout except as stated below; diet 13 from beginning of experiment; ↑ diet 25; diet 29; ⊙ restricted food intake; × food *ad libitum*; ↕ diet 44, B_1 supplement stopped; ψ diet 49 (no B_1).

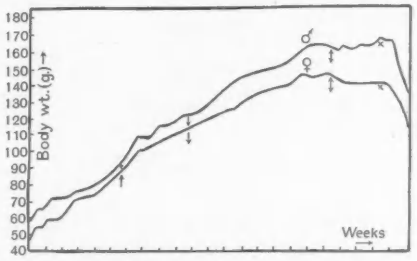

Fig. 9. Dose of vitamin B_1 = 0·075 cc. of lead filtrate throughout except as stated below; basal diet 14 from beginning of experiment; ↑ diet 26; ↓ diet 30; ↕ diet 44, B_1 supplement stopped; × diet 49 (with no B_1).

kept on a restricted amount of food for 18 days (6 g. per day for the first 3 days and 8 g. daily during the rest of the period). The changes in weight are shown in Table V.

Table V.

Rat No.	Sex	Diet	Period (days)	Initial weight (g.)	Final weight (g.)	Gain in weight in 18 days (g.)
1	♂	28	18	155	157	+ 2
2	♂	29	18	158	180	+22
3	♀	28	18	128	130	+ 2
4	♀	29	18	148	172	+24

This clearly shows that the diet containing 40 % lard promotes faster growth under the above conditions than that containing 10 % lard. Lard seems to be in this respect considerably more effective than palm kernel oil or olive oil.

Vitamin B_2, fed in the form of alkaline-autoclaved marmite, does not appear to have any effect [see also Evans and Lepkovsky, 1929].

D. The effect of the ingestion and injection of sodium lactate in vitamin B_1 deficiency.

As the accumulation of lactic acid has been stated to be concerned in the production of both avian and human "beriberi" [Peters, 1930; Hayasaka, 1930] it was considered of interest to investigate the effect of ingested sodium lactate on the rate of decline in weight and on the production of "beriberi" symptoms in vitamin B_1-deficient rats. Experiments were also carried out to observe the effect of the subcutaneous injection of sodium lactate in varying doses into deficient rats.

In this investigation the following diets were used:

Diet 21.	Sucrose	65 parts
	Palm kernel oil	10 ,,
	"Light, white casein"	20 ,,
	Salt mixture (McCollum)	5 ,,
	Cod-liver oil	2·5 ,,
	50 % alkalised marmite	10 cc.

Diet 23 was made up by incorporating 10 cc. of a vitamin B_1 preparation (lead filtrate) per 100 g. of diet 21 so that 10 g. of the diet contained 1·5–2 rat day doses.

Diet 38 was made up by incorporating 10 cc. of 50 % sodium lactate per 100 g. of diet 21.

(*a*) *The ingestion of sodium lactate.*

The method consisted in feeding two groups of adult rats, grown to maturity on whole wheat, brown bread and milk, on diets 21 and 38 respectively and comparing their rates of decline in weight and noting the time of onset of convulsions. Table VI summarises some of the results obtained.

The average rates at which the animals on diets 21 and 38 lost weight under the conditions studied were 8·0 and 12·5 weekly. Although this would tend to indicate a more rapid loss in weight when lactate was present

Table VI.

Rat No.	Sex	Diet No.	Period of feeding on synthetic diet (weeks)	Initial weight (g.)	Final weight (g.)	Total loss in weight (g.)	Loss in weight per week (g.)	Remarks
1	♂	21	6	131	116	15	2·5	Convulsions on 45th day
2	♂	21	6	247	165	82	13·7	Convulsions on 45th day
3	♂	21	6	221	158	63	10·5	Convulsions on 49th day
4	♂	21	6·5	165	148	17	2·6	—
5	♂	21	5·5	135	105	30	5·4	Convulsions on 44th day
6	♂	21	4·5	160	90	70	15·6	Convulsions on 29th day
7	♂	21	3	192	143	49	13·0	Had been on deficient diet for 5 weeks previously; convulsions on 56th day
8	♂	21	8	183	136	47	5·9	Convulsions on 56th day
9	♂	21	11	162	120	42	3·8	Dead on 78th day
10	♂	21	12	207	121	86	7·1	Convulsions on 85th day
11	♂	38	8	172	100	72	9·0	Convulsions on 57th day
12	♂	38	8	193	119	74	9·2	Convulsions on 57th day
13	♂	38	8	222	150	72	9·0	Convulsions on 61st day
14	♂	38	8	240	150	90	11·2	Convulsions on 61st day
15	♂	38	6	187	100	87	14·5	Convulsions on 43rd day
16	♂	38	6·5	178	120	58	9·0	—
17	♂	38	5	188	102	86	17·2	Convulsions on 43rd day
18	♂	38	3	241	178	63	21·0	Had been previously on diet 21; convulsions on 61st day

in the diet, the large individual variations among the members of each group of rats preclude too great a reliance on these figures. It has also to be noted that the presence of lactate in the diet did not hasten the appearance of the "beriberi" convulsions. But here also the values are too scattered to warrant a definite conclusion.

As the metabolism of sodium lactate was likely to be reflected in the hydrogen ion concentration of the urine, the p_H values of the urine of rats on diets 21, 23 and 38, diet 38 + vitamin B_1, and on normal stock diet were measured and are given in Table VII. The urine was collected for 5 hours over toluene.

Table VII.

Rat No.	Sex	Diet	Nature of diet	p_H of urine
1	♀	Normal stock diet	Whole wheat, brown bread and milk	6·5
2	♂	21	〃	6·5
3	♂	23	+ vitamin B_1	6·4
4	♂	23	+ 〃	6·5
5	♂	23	+ 〃	6·5
6	♂	21	− 〃	6·2
7	♂	21	− 〃	6·5
8	♂	21	− 〃	6·4
9	♂	38	− vitamin B_1; + lactate	7·2
10	♂	38	〃	7·2
11	♂	38	〃	7·2
12	♀	38 + vitamin B_1	+ vitamin B_1; + lactate	8·6
13	♀	〃	〃	8·6
14	♂	〃	〃	8·7

These results show definitely that while the presence of sodium lactate in the vitamin B_1-deficient diet raises the p_H of the urine from approximately

6·4 to 7·2, the addition of vitamin B_1 to the lactate diet raises the p_H to 8·7. This rise in p_H occurs within 24 hours of the administration of vitamin B_1 to the basal lactate diet.

One rat, weighing 212 g., which was on diet 23, was injected with 2 cc. of 50 % sodium lactate. The p_H values of samples of the urine collected successively after 18 hours and 48 hours were 9·2 and 7·8. The urine collected after 7 days gave the p_H value 6·6, showing a return to the normal.

The significance of these results is discussed later.

(b) The injection of sodium lactate.

The results obtained by the subcutaneous injection of sodium lactate into rats on diets 21 and 23 are summarised in Table VIII. The animals on diet 21 were subjected to a fairly severe deficiency of vitamin B_1, but the deprivation was not sufficiently prolonged to allow them to go into "beriberi" convulsions spontaneously.

Table VIII.

Rat No.	Sex	Diet No.	Nature of diet	Period of feeding on synthetic diet (days)	Body weight at time of injection (g.)	Dose of 50 % sodium lactate injected (cc.)	Results
1	♂	21	− vitamin B_1	45	148	0·2	Survived
2	♀	21	− ,,	52	60	0·4	Survived
3	♀	21	− ,,	54	55	0·4	Died in 4 hours in convulsions
4	♀	21	− ,,	50	90	0·6	Survived
5	♀	21	− ,,	59	70	0·6	Died in an hour
6	♂	21	− ,,	50	95	0·8	Died in 4 hours in convulsions
7	♀	21	− ,,	58	94	0·8	Died in 1½ hours in convulsions; signs of haemorrhage
8	♀	21	− ,,	23	85	0·8	Survived; severe convulsions after 4 days
9	♂	21	− ,,	56	148	1·0	Survived
10	♂	21	− ,,	46	150	1·0	Survived
11	♀	23	+ ,,	59	174	0·8	Survived
12	♀	23	+ ,,	56	105	1·0	Survived
13	♀	23	+ ,,	49	118	1·0	Survived
14	♂	23	+ ,,	49	107	1·6	Died after 5 hours
15	♂	23	+ ,,	61	225	1·6	Survived
16	♂	23	+ ,,	50	142	1·8	Died after 6 hours
17	♀	23	+ ,,	58	122	1·8	Survived
18	♀	23	+ ,,	87	175	2·0	Survived
19	♂	23	+ ,,	49	152	2·0	Died in 3 hours
20	♀	23	+ ,,	56	150	2·0	Survived
21	♂	23	+ ,,	56	185	2·4	Survived
22	♂	23	+ ,,	49	108	2·4	Died in 3 hours
23	♂	23	+ ,,	63	225	2·4	Survived
24	♂	23	+ ,,	64	225	3·2	Convulsions; severe haemorrhage; died in 1 hour

These figures demonstrate that while a dose of about 0·8–1·0 cc. of 50 % sodium lactate per 100 g. body weight is fatal for vitamin B_1-deficient rats, animals receiving vitamin B_1 exhibit a tolerance at least one and a half times as great.

The convulsions observed in the vitamin B_1-deficient animals on injection of sodium lactate sometimes resembled typical "beriberi" convulsions. Attempts were made to find if these artificial convulsions could be cured by the administration of vitamin B_1 preparations, but death was too quick to be prevented by this means.

As the "beriberi" symptoms in pigeons have been ascribed to acidosis produced by lactic acid [Peters, 1930], it was of interest to compare the effects of the injection of similar doses of ammonium chloride, which is known to produce marked acidosis [Salkowski, 1878; Haldane, 1921], into vitamin B_1-deficient and normal rats. The quantities of ammonium chloride injected were approximately equimolecular to some of the smaller doses of sodium lactate used in the preceding set of experiments. Ammonium chloride was, however, found to be much more toxic than sodium lactate and appeared to be equally fatal to normal and deficient rats, in the doses tried (Table IX).

Table IX.

Rat No.	Sex	Diet	Period on synthetic diet (days)	Body weight at time of injection (g.)	Dose of 22·2 % sol. of NH_4Cl injected (cc.)	Results
1	♂	21	56	90	0·9	Died in 15 minutes
2	♂	21	49	80	0·45	Died in 15 minutes
3	♀	21	—	100	0·9	Died in 10 minutes
4	♀	21	—	147	0·9	Died in 15 minutes
5	♂	23	56	198	1·8	Died in 5 minutes (severe haemorrhage)
6	♂	23	56	200	0·9	Died in 20 minutes
7	♀	23	57	170	0·9	Died in 15 minutes

E. The oxidative mechanism of tissues in vitamin B_1-deficiency.

Suggestions regarding the impairment of the oxidative apparatus in "vitamin B" deficiency are numerous. Drummond and Marrian [1926, where the literature up to 1926 is quoted], from a series of experiments carried out with rats, found, however, no experimental support for the theory. The question has been very recently revived by Abderhalden and Vlassopoulos [1931] working with the liver, muscle and brain tissues of pigeons.

The present work was undertaken in view of the statement of Peters [1930] regarding an increase in the production of lactic acid in vitamin B_1 deficiency in the pigeon and its concentration in the brain, and of the recent experiments of Roche [1931], which also tend to show an accumulation of three-carbon bodies in the blood of avitaminous pigeons. It was considered, firstly, that if in vitamin B_1 deficiency the specific mechanism concerned in the removal of lactic acid was involved, its impairment might not be revealed by the technique employed by Drummond and Marrian, who used succinate as the metabolite in their anaerobic reduction experiments with methylene blue. The succino-dehydrogenase system might be unimpaired in vitamin B_1 deficiency, while the lactic dehydrogenase system might be damaged. Secondly, the experiments

on the direct oxygen uptake of tissues might not lead to any definite conclusions on this question, because the lactic-oxidising system, while being qualitatively important, might not be quantitatively so great in relation to the total uptake that its impairment would cause a markedly diminished oxygen consumption. This diminution would have to be sufficiently large to be outside the error caused by the fortuitous presence of various metabolites in the particular tissue examined at the moment of killing the animal. As in such experiments the oxygen uptakes of the tissues of different animals have to be compared, the variation introduced by this factor is likely to be large. A more correct idea of the condition of the lactic-oxidising system would be obtained by measuring the increased oxygen uptake due to added lactate, though there might not be a strictly proportional relation between the two owing to the competition of different substrates being involved in the question. Thirdly, since Kinnersley and Peters [1930] have shown that lactic acid accumulates chiefly in the brain, it was considered possible that while other "beriberi" tissues might not show any marked difference in oxygen consumption from normal tissues, the brain might reveal the difference. The liver might also be concerned in this question, as it apparently plays an important part in lactic acid metabolism [Cori and Cori, 1929].

In the following experiments, therefore, which were carried out throughout with the brain and liver tissues of rats, the significant values are considered to be those that were obtained after the addition of sodium lactate as substrate, both in the anaerobic experiments with methylene blue and the experiments on direct oxygen consumption.

(1) *Experiments with Thunberg's methylene blue technique.*

The Thunberg tubes were arranged according to the following general scheme.

	Tubes without lactate cc.	Tubes with lactate cc.
Phosphate buffer (p_H 7·4)	2·0	2·0
Methylene blue (1/5000)	1·0	1·0
Na lactate (M/60)	—	0·5
Distilled water	2·0	1·5
Tissue suspension in phosphate buffer	1·0	1·0

Temp. of bath 37°.

The results of certain typical experiments with liver and brain tissues of normal and vitamin B_1-deficient animals are shown in Table X.

Figures of a similar order were obtained in other experiments. Thus, with 0·148 g. of the brain tissue of one deficient rat the time of reduction of methylene blue in presence of sodium lactate was 4·1 minutes, the reduction time without lactate being 18·2 minutes, showing a fair degree of efficiency of the mechanism concerned in the oxidation of lactic acid.

Though both the liver and brain tissues of normal animals reduce methylene blue more rapidly than the corresponding tissues of vitamin B_1-deficient

Table X.

Liver tissue.

		Time of decoloration without lactate (mins.)		Time of decoloration with lactate (mins.)	
Unwashed liver tissue (0·15 g.)	Normal	5·5 5·75 5·7	5·65	5·0 4·05 4·15	4·4
	Vitamin B_1-deficient	11·6 9·25 10·0	10·28	6·6 7·2 7·2	7·0
Washed liver tissue* (0·15 g.)	Normal	Slight decoloration in 3 hours		35·2 40·25	37·72
	Vitamin B_1-deficient	,,		49·0 46·0	47·5
	Brain tissue.				
Washed brain tissue† (0·14 g.)	Normal	26·1 24·0 24·4	24·8	6·1 5·6 5·9	5·87
	Vitamin B_1-deficient	37·25 38·0 37·5	37·58	7·0 6·9 6·8	6·9

* Washed twice with 100 cc. of Ringer-Locke solution per 1·5 g. of tissue. Too much washing apparently causes a large loss of lactic dehydrogenase.

† Washed once with 100 cc. Ringer-Locke solution per 1·4 g. tissue.

animals in the absence of sodium lactate (Table X), no valid conclusion regarding the oxidative mechanism can be drawn from these figures, as has been pointed out earlier. More significant values are obtained by calculating the ratio (a:b) of the time of reduction of methylene blue without lactate to the time of reduction with lactate in each case (Table XI).

Table XI.

	a : b
Liver tissue of normal rat	1·30
,, deficient rat	1·47
Brain tissue of normal rat	4·22
,, deficient rat	5·44
,, ,,	4·50

These ratios indicate that the acceleration of the reduction of methylene blue due to added lactate by normal and deficient liver and brain tissues is approximately of the same order for each type of tissue. The variations fall within the limits of error of the technique.

(2) *Experiments on the oxygen consumption with the Barcroft apparatus.*

These experiments were carried out with the chopped whole brain tissue (unwashed).

The general arrangement of the cups is shown in Table XII. Temp. 38°.

Table XII.

	Without lactate		With lactate	
	Left-hand cup cc.	Right-hand cup cc.	Left-hand cup cc.	Right-hand cup cc.
Phosphate buffer (p_H 7·2)	1·0	1·0	1·0	1·0
Sodium lactate ($M/10$)	—	—	—	0·5
Ringer solution	1·5	0·5	1·5	0·5
Distilled water	0·5	0·5	0·5	—
Brain tissue suspension in phosphate buffer	—	1·0	—	1·0

Some results are shown in Figs. 10 and 11 which represent extreme values. Thus in one case the rate of oxygen consumption of the normal tissue was

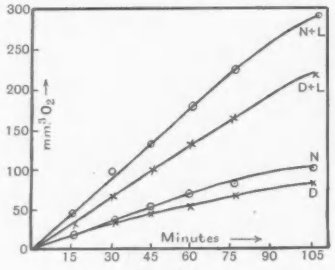

Fig. 10.

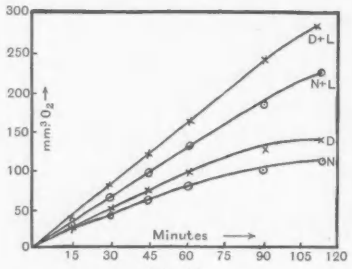

Fig. 11.

Fig. 10. Oxygen consumption of 0·1 g. brain tissue.
×—×—× Tissue of deficient animal in convulsions (D).
⊙—⊙—⊙ Tissue of normal animal (N).
D + L stands for "beriberi" tissue with added lactate.
N + L stands for normal tissue with added lactate.

Fig. 11. Oxygen consumption of 0·13 g. brain tissue.
×—×—× Tissue of deficient animal (D).
⊙—⊙—⊙ Tissue of normal animal (N).
D + L stands for "beriberi" tissue with added lactate.
N + L stands for normal tissue with added lactate.

somewhat greater than that of the "beriberi" tissue, while in the other case the situation was reversed, though this latter case was somewhat exceptional.

Addition of lactate as substrate resulted in a very marked increase in the rate of oxygen uptake of both the normal and "beriberi" tissues. The orders of increase, however, show some variations, and it appears that this technique would not reveal any but a considerable damage to the lactic-oxidising mechanism in vitamin B_1 deficiency, unless a statistical analysis of a very large number of results were made.

The cytochrome content of normal and "beriberi" tissues.

The brain, liver, kidney and heart muscle tissues of normal rats and vitamin B_1-deficient rats in convulsions (which were nearly killed by chloroform and then bled to death) were thoroughly washed and examined for the cytochrome bands in the usual way with the microspectroscope, after the addition

of a trace of sodium hydrosulphite, if necessary. All the cytochrome bands were equally prominent in the corresponding tissues of normal and avitaminous animals. There was, apparently, no diminution of the cytochrome content in vitamin B_1 deficiency.

Discussion.

The experiments described in Section A offer no support to the contention of Randoin and Simonnet [1924] that the requirement of vitamin B_1 has a relation to the carbohydrate content of the diet of pigeons. On the other hand, it has to be pointed out that these experiments on the relation between vitamin B_1 and the protein/carbohydrate ratio of the diet constitute no evidence against the theory that vitamin B_1 plays some part in carbohydrate metabolism, as it is quite possible that the vitamin is required also for the degradation of the glucogenic residue of the protein molecule. The non-dependence of the requirement of vitamin B_1 on the nature of the dietary carbohydrate (glucose, fructose, sucrose and maltose) is not probably of much significance, because presumably all these carbohydrates yield the same three-carbon compounds by degradation and, if vitamin B_1 is concerned in the metabolism of the latter [Peters, 1930; Vogt-Møller, 1931; Roche, 1931] it is quite reasonable that the requirement of vitamin B_1 should be independent of the nature of the carbohydrate. The decline in weight of animals receiving galactose as the sole source of carbohydrate is probably to be related to its inefficient utilisation and its low renal threshold. Its absorption through the intestinal wall is known to be rapid [Cori, 1925; McCance and Madders, 1930]. The lactose diet had a somewhat deleterious effect in that it tended to produce diarrhoea.

The experiments on the relation between vitamin B_1 and dietary fat indicate that all fats are not of equal value so far as the sparing action on vitamin B_1 is concerned. Thus lard is apparently more efficient in this respect than palm kernel oil and olive oil. It is, therefore, not clear how far the sparing action is the effect of fat *per se*. While the observations of Evans and Lepkovsky [1929] on this subject might be interpreted to mean that the vitamin plays some rôle in carbohydrate metabolism, other possible explanations might also be suggested. Thus, assuming that the vitamin acts only by stimulating the appetite in some unknown way, it is possible that in Evans and Lepkovsky's experiments, if the limiting factor in the growth of the deficient rats were calorie intake, the rats receiving fat-rich diets would grow better because of the relatively much greater calorie intake. The observation mentioned in this paper, that rats receiving restricted amounts of a diet containing 40 % lard grew better than those receiving the same quantities of a diet containing 10 % lard when both groups were given a sub-optimum dose of vitamin B_1, might also be interpreted in a similar manner. The phenomenon of refection reminds one also of a possible function of the intestinal bacteria of rats subsisting on high-fat diets. It may be mentioned incidentally that the high-fat diets did not exert any deleterious effect on the animals [see also Osborne

and Mendel, 1924], except that they were greasy looking and somewhat sluggish.

As described in Section D the appearance of symptoms was not markedly hastened by the presence of sodium lactate in a vitamin B_1-deficient diet. The absorption of sodium lactate is known to be fairly rapid [Cori, 1930] and the change in p_H of the urine indicates that the ingested sodium lactate was being metabolised. The expectation that sodium lactate might precipitate the appearance of symptoms in deficient rats was based on the observation of Kinnersley and Peters [1930] regarding the accumulation of lactic acid in the brain and on the evidence presented by McGinty [1929] to show that brain tissue normally removes some of the lactic acid from circulation. The p_H (8·4) of the urine of rats receiving the lactate diet *plus* vitamin B_1, which was higher than the p_H (7·2) of the urine of rats receiving the lactate diet *minus* vitamin B_1, might be interpreted to indicate a greater efficiency of the animals receiving vitamin B_1 in metabolising ingested lactate. This phenomenon might, however, also be ascribed to the diminished consumption of the lactate diet in vitamin B_1 deficiency. How far the ability to dispose of lactic acid is affected in vitamin B_1 deficiency is at present under investigation in experiments both *in vivo* and *in vitro*.

The injection experiments with sodium lactate showed a definitely decreased tolerance to lactate in vitamin B_1 deficiency. It is possible, however, that this was due to the comparatively weak condition of the deficient rats. The convulsions induced by the injection of lactate sometimes resembled "beriberi" convulsions, but insulin convulsions are also similar in appearance.

The significant evidence brought forward by Kinnersley and Peters [1929, 1930] regarding an increase in blood-lactic acid and a localised lactic acidosis in the brain finds support in the recent experiments of Roche [1931] on the blood of deficient pigeons, and of Hayasaka [1930], who observed that sodium lactate, injected intravenously into normal and cured "beriberi" human subjects, disappeared faster than that injected into "beriberi" subjects. As has been indicated earlier, the evidence for the depressed oxidation of various tissues of deficient pigeons [Abderhalden and Vlassopoulos, 1931], and for the diminished respiration of the blood of "beriberi" subjects [Kimura, 1930], might be misleading unless the differences are of a relatively high order, and unless a very large number of measurements are taken. In a recent note, which appeared after the present work had been completed, Gavrilescu and Peters [1931] have recorded the interesting observation that the lowering of the oxygen consumption of the brain tissue of avitaminous pigeons was confined especially to certain parts of the brain. If such be the case, it might have escaped detection in the experiments described in Section E, which were carried out with the whole brain tissue of rats. These experiments on the increased oxidation due to added lactate indicate, however, that the mechanism concerned in the oxidative removal of the particular metabolite, lactate, is not seriously damaged in vitamin B_1 deficiency. While it is possible that a

statistical analysis of a large number of measurements might indicate a small amount of injury to this mechanism, it is considered probable that, so far as the lactic acid observed to accumulate by Kinnersley and Peters is concerned, it is the mechanism of its "synthetic" removal which is damaged rather than that of its oxidative removal. Holmes and Ashford [1930] have demonstrated the existence of a Meyerhof quotient for the brain, though at the moment no information is available as to the fate of the lactic acid which is removed but not oxidised by brain tissue. In fact, our knowledge about the steps by which the lactic acid in muscle is resynthesised into glycogen, is also very meagre [Long and Grant, 1930]. Investigation is in progress to test the accuracy of the above suggestion.

Summary.

1. The requirement of vitamin B_1 for young growing rats, receiving a sub-optimum dose of vitamin B_1, was found to be independent of the protein/carbohydrate ratio of the diet.

2. The requirement of vitamin B_1 under the same conditions was likewise independent of the nature of carbohydrate in the diet, glucose, fructose, sucrose or maltose. With galactose and lactose as sole sources of carbohydrate in the diet the animals were found to decline in weight.

3. The relation of the requirement of vitamin B_1 to the dietary fat has been investigated. While palm kernel oil and olive oil had little sparing action on the vitamin, lard appeared to have a definite effect.

4. The ingestion of sodium lactate did not appreciably hasten the appearance of symptoms in vitamin B_1-deficient rats. The p_H of the urine of animals receiving lactate *plus* vitamin B_1 was higher than that of animals receiving lactate *minus* vitamin B_1. The lethal dose of injected sodium lactate was lower for deficient animals than for normal ones.

5. The cytochrome content of the liver, kidney, brain and heart muscle tissues of rats was not diminished in vitamin B_1 deficiency. The mechanism for the oxidation of lactic acid by the liver and brain tissues of deficient rats appeared to remain fairly efficient.

I am indebted to Sir F. G. Hopkins for his interest in this work and to Dr Keilin and Dr Cook for generous help at certain stages of this investigation. To Miss R. Leader my thanks are due for able assistance with the animals.

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CL. ON GALACTOSE AS THE DIETARY CARBOHYDRATE.

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(*Received July 3rd, 1931.*)

In course of an investigation on a possible relationship between the requirement of vitamin B_1 and the nature of the dietary carbohydrate [Guha, 1931] it was observed that if galactose formed the sole carbohydrate in a fat-free diet the rats rapidly declined in weight and died. When young rats of about 50 g. in weight were used, death occurred within 3–4 days. Uniform results were obtained with galactose (extra pure) of the British Drug Houses and with Kahlbaum's product. Though the utilisability of galactose is known to be relatively poor, this unique behaviour of galactose among the carbohydrates studied merited further attention, especially as such studies might have some relation to the still unsolved problem of the significance of the occurrence of lactose in milk. In the present investigation the effect of a galactose diet unsupplemented by any other adequate source of non-protein energy on young and adult rats has been studied. Experiments have also been carried out to find how far the ill-effects observed could be averted by the presence of varying amounts of glucose, fat and protein in the diet. The behaviour of mice under similar circumstances has also been studied in a few preliminary experiments. The results of the work, though incomplete, are published at this stage as the investigation cannot be pursued further at the moment.

Experimental.

The rats used were mostly of the black and white strain, a few being albinos. For comparative experiments litter-mates were employed. As usual, the animals were kept in separate cages and all vitamin supplements were fed separately from the basal diet except when yeast was used. The vitamin B_1 and B_2 preparations used were the same as employed in the work described in the preceding communication [Guha, 1931]. Each animal received 2 drops of cod-liver oil daily. In several cases records of the food and water intake and of the urinary output were kept.

The following diets were used. The caseinogen used was the "light, white" variety of the British Drug Houses.

[1] Travelling Fellow of the University of Calcutta.

Diet 19	Parts
Galactose	75
Caseinogen	21
Salt mixture (McCollum)	4

Diet 19 (*a*)	Parts
Galactose	37·5
Glucose	37·5
Caseinogen	21
Salt mixture	4

Diet 19 (*b*)	Parts
Galactose	37·5
Palm kernel oil	37·5
Caseinogen	21
Salt mixture	4

Diet 19 (*c*)	
Galactose	70
Caseinogen	21
Dried yeast	5
Salt mixture	4

Diet 19 (*d*)	
Galactose	35
Glucose	35
Caseinogen	21
Dried yeast	5
Salt mixture	4

Diet 19 (*f*)	
Glucose	70
Caseinogen	21
Dried yeast	5
Salt mixture	4

Diet 19 (*g*)	
Galactose	60
Palm kernel oil	16
Caseinogen	21
Salt mixture	4

Diet 19 (*h*)	
Galactose	35
Caseinogen	56
Dried yeast	5
Salt mixture	4

Diet 19 (*j*)	
Galactose	70
Caseinogen	19
Sodium dihydrogen phosphate	2
Dried yeast	5
Salt mixture	4

Diet 19 (*k*)	
Galactose	70
Palm kernel oil	10
Caseinogen	21
Salt mixture	4

Diet 19 (*l*)	
Galactose	70
Palm kernel oil	5
Caseinogen	21
Salt mixture	4

Diet 1	
Glucose	75
Caseinogen	21
Salt mixture	4

A. *Experiments with adult rats.*

Rats which had been growing for a considerable period on diet 1, containing glucose supplemented by vitamin B_1 and B_2 preparations and cod-liver oil, were fed on diet 19 with the same supplements. The rats rapidly declined and died (Fig. 1).

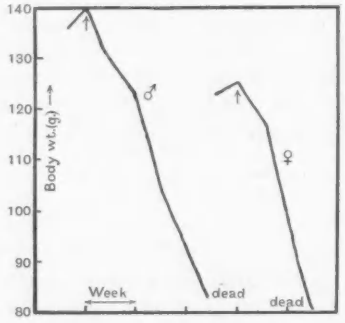

Fig. 1. ↑ Diet 19 supplemented with vitamin B_1 and B_2 preparations.

B. *Experiments with young rats.*

Litters of young rats were broken up into groups of two. The behaviour of these groups on diets 19 (*c*) containing galactose, 19 (*f*) containing glucose, 19 (*d*) containing equal parts of glucose and galactose, 19 (*j*) containing galactose with extra phosphate and 19 (*h*) containing galactose with extra caseinogen

is illustrated in Figs. 2 and 3. All the diets contained 5 % yeast as the source of "vitamin B," which contributed to a small extent to the energy value of

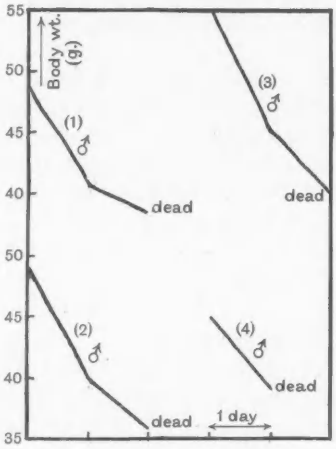

Fig. 2.

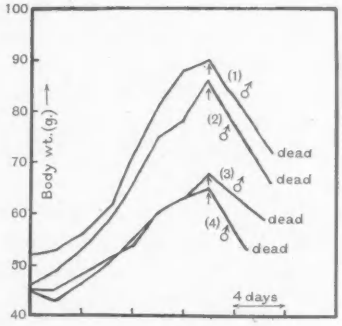

Fig. 3.

Fig. 2. Curve (1). Diet 19 (*c*). Curve (2). Diet 19 (*c*). Curve (3). Diet 19 (*h*). Curve (4). Diet 19 (*h*).

Fig. 3. Curve (1). Diet 19 (*f*) from beginning of experiment; ↑ diet 19 (*c*).
Curve (2). Diet 19 (*f*) from beginning of experiment; ↑ diet 19 (*c*).
Curve (3). Diet 19 (*d*) from beginning of experiment; ↑ diet 19 (*j*).
Curve (4). Diet 19 (*d*) from beginning of experiment; ↑ diet 19 (*j*).

the diets. In diet 19 (*j*) extra phosphate was incorporated to find if it had any influence on the utilisation of galactose. Diets 19 (*c*), 19 (*j*) and 19 (*h*) were unable to support life, while the animals receiving diet 19 (*d*) grew at a markedly slower rate than those receiving diet 19 (*f*). This last case shows that 35 % glucose cannot support the normal growth of young rats and that the same quantity of galactose cannot replace it in the diet.

(*a*) *Food and water intake.* In Table I are given the figures for the food and water intakes of some animals receiving diets 19 (*f*) and 19 (*d*). Although the animals receiving diet 19 (*d*) were growing more slowly than those receiving diet 19 (*f*), the former were actually consuming more food than the latter. The water intake was also considerably higher.

Table I.

Rat No.	Sex	Diet	Period over which food intake records were kept (days)	Average daily food intake (g.)	Average daily water intake (cc.)
1	♂	19 (*f*)	10	9·9	22·6
2	♂	19 (*f*)	15	10·6	17·8
3	♂	19 (*d*)	10	12·6	31·2
4	♂	19 (*d*)	15	12·8	34·8

The animals receiving diet 19 (*c*) consumed larger quantities of food and took much more water than those of any other group. It was difficult, however, to keep accurate food intake records owing to the scattering of the diet. The urinary output of these animals was also considerably higher, reaching as much as 40–45 cc. daily.

(*b*) *Condition of the animals.* The rats on diet 19 (*c*) soon lost the lustre of the eye and became hunched. The fur became ruffled and other signs of weakness resembling those observed in vitamin B_1-deficient rats were also present. The animals lay sometimes in a comatose condition with extended limbs for 20 hours before death. In this condition there was no appreciable response to two injections of a total volume of 1·2 cc. of 10 % glucose.

Post mortem examination, kindly carried out by Mr J. R. M. Innes, revealed no organic lesions. The intestinal tract had an unhealthy appearance.

C. *Experiments with young rats.*

In Fig. 4 are illustrated the growth curves of young rats on diets 1, 19, 19 (*a*), 19 (*b*), 19 (*g*), 19 (*k*), 19 (*l*) and 19 (*c*). Of these diets all except diet

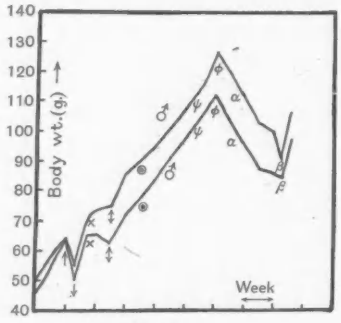

Fig. 4.

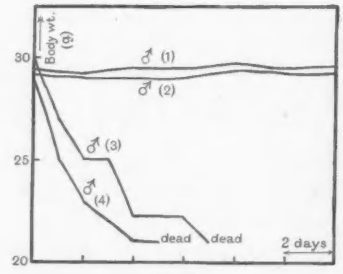

Fig. 5.

Fig. 4. Diet 1 from beginning of experiment; ↑ diet 19; ↓ diet I; × diet 19 (*a*); ‡ diet 19 (*b*); ⊙ diet 19 (*g*); ψ diet 19 (*k*); ϕ diet 19 (*l*); α diet 19 (*c*); β "complete" synthetic diet.

Fig. 5. Experiments with mice. Curve (1). Diet 19 (*f*). Curve (2). Diet 19 (*f*). Curve (3). Diet 19 (*c*). Curve (4). Diet 19 (*c*).

19 (*c*), which contained yeast, were supplemented daily by 1 cc. of a vitamin B_1 concentrate and 1 cc. of a vitamin B_2 preparation, so as to eliminate as far as possible the source of energy provided by the glycogen, fat and protein of yeast. Diet 19 (*g*) was made up so as to contain an amount of palm kernel oil approximately isodynamic with 37·5 g. glucose. This was done in order to compare the effects of this diet and of diet 19 (*a*) which are calorifically approximately equivalent, neglecting galactose as a source of energy. It will be observed that diet 19 (*g*) has a distinctly better effect. It will also be noticed that, by gradually lowering the content of fat in the galactose diet, growth can be progressively inhibited. (Compare diets 19 (*b*), 19 (*g*), 19 (*k*)

and 19 (*l*).) Food intake records were kept throughout this set of experiments, which show that the greatest food consumption occurred with diet 19 (*c*), approaching sometimes 23 g. daily.

D. *Experiments with mice.*

Only a few preliminary experiments could be carried out with mice[1]. These have yielded results similar to those obtained with rats. These animals, which were nearly adult, were fed on diets 19 (*f*) and 19 (*c*). The results are illustrated in Fig. 5. It will be observed that, while the glucose diet was apparently adequate, the galactose diet failed to support life.

The striking fact about the mice receiving diet 19 (*c*) was that they exhibited all the symptoms typical of "beriberi" in the rat in addition to those observed in the rats on the same diet. Thus, after 5–7 days on this diet there was loss of muscular co-ordination and they went into convulsions when swung, which were indistinguishable from "beriberi" convulsions in the rat. The animals could remain in the moribund condition for over 40 hours. It has to be mentioned that they were eating considerable quantities of food for the first three days and were, therefore, receiving ample quantities of yeast from the diet.

Discussion.

The results described indicate that the degree to which galactose can be utilised by the rat is singularly low. Death can be prevented by replacing part of the galactose by glucose or by fat but not by increasing the protein content of the diet. That the limiting factor in the galactose diet is its inability to supply sufficient energy to the rat is also indicated by the proportionate growth responses to graded amounts of fat in the galactose diet. The increased water intake and diuresis on the galactose diet is readily intelligible in the light of our knowledge about the rapid absorption of galactose through the intestinal wall [Cori, 1925; McCance and Madders, 1930] and of its low renal threshold [Folin and Berglund, 1922]. In fact, this appears to be the reason why death is so quick in young animals on the galactose diet. As has been pointed out, the food intake on this diet is greater than on any other, which indicates that the low utilisability of galactose prompts the consumption of more food and, as galactose is quickly absorbed and cannot be readily oxidised or converted into glycogen [Lusk, 1915; Weinland, 1899; Ishimori, 1912–13], it has to be excreted. Thus, the whole process of digestion, absorption and excretion involves a greater strain than would have been the case if the animal were only starving and, therefore, apparently precipitates death. In this matter these animals behave differently from vitamin B_1-deficient rats, because, while the former continue to consume food till shortly before death and undergo virtual but not actual starvation, the vitamin B_1-deficient animals, on the other hand, starve themselves voluntarily. Attention has to

[1] I am indebted to Miss E. Turner for supplying me with these animals.

be drawn to the fact that many of the symptoms are common to both groups of animals, which, again, supports the contention that in vitamin B_1 deficiency the usual symptoms are simply those of starvation, consequent on anorexia. The typical symptoms of "beriberi" observed in mice subsisting on the galactose diet and receiving ample "vitamin B" also indicate the non-specificity of the so-called "beriberi" symptoms.

These experiments make the question of the function of lactose in nutrition still more puzzling. While Folin and Berglund [1922] have shown that the utilisation of galactose is apparently increased in presence of glucose, the present investigation indicates the desirability of the study of the rôle of fat in this connection. It appears from this study that the energy value of the fat in milk might be nearly as important for the nutrition of the young as are the vitamins it carries.

SUMMARY.

Young and adult rats on a fat-free diet containing galactose as the sole carbohydrate rapidly decline in weight and die. The symptoms of these animals are described. Mice behave similarly under the same conditions and develop typical "beriberi" symptoms although receiving ample quantities of "vitamin B." How far growth can be supported in young rats by incorporating varying quantities of glucose, fat and protein in the galactose diet has been studied.

My thanks are due to Sir F. G. Hopkins for criticism and advice.

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CLI. THE CHEMICAL NATURE OF VITAMIN B_1 FROM EVIDENCE AFFORDED BY ITS ELECTRICAL TRANSFERENCE.

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(*Received July 2nd, 1931.*)

Since the recognition of the dietary principle now known as vitamin B_1 a great number of attempts have been made to isolate it during the last thirty years. Though nearly all these attempts have admittedly failed in their main objective, the methods of fractionation generally employed suggest that the vitamin is probably of a basic nature. This conclusion is, however, complicated by the probability that adsorption plays a more important rôle in these fractionations than does true chemical reaction. Moreover, ample evidence has been brought forward by several investigators [Kinnersley and Peters, 1928; Guha and Drummond, 1929] to show that the behaviour of vitamin B_1 is to a large extent modified by the associated substances present. The adsorption of vitamin B_1 by fuller's earth and "acid clay," however, is apparently of the polar type and, therefore, strengthens the supposition that vitamin B_1 is a base. All this evidence, however, is indirect. The present paper records the results of experiments on the behaviour of various preparations of vitamin B_1, as also of crude yeast extracts, on being subjected to electrodialysis at different hydrogen ion concentrations. This investigation had three objects in view:

(1) to obtain what might be regarded as direct and conclusive evidence as to the basic nature of vitamin B_1 and to attempt to find the approximate p_K value of its basic group;

(2) to examine the possibilities of concentration of vitamin B_1 by this method;

(3) to attempt a separation of vitamin B_1 from other members of the vitamin B complex.

After this work had been completed, our attention was drawn to a paper by Williams and Waterman [1929], in which they discuss the possibility of the separation of ampholytes by electrodialysis using a multiple compartment cell. They state that "the two factors which can be recognised most definitely by our present feeding technique migrate towards the alkaline region where they undergo fairly rapid decomposition. These factors are the antineuritic

[1] Travelling Fellow of the University of Calcutta.

vitamin and the one designated as 'the third factor.'" No details, however, are given. Professor Drummond has also recently informed us of the unpublished work of A. F. Watson in his laboratory in 1921, who observed with crude yeast extracts that "vitamin B" (its complex nature not being established at that time) migrated towards the cathode at p_H 3·8.

Experimental.

A three compartment cell was used for the electrolysis. It was made by cutting a wooden box, open at the top and of suitable dimensions, into three vertical sections of equal length. Each section was soaked in paraffin-wax to render it impermeable to water. Four rubber washers were cut to fit the ends of the sections, a parchment membrane was placed between each pair so as to separate the three sections, and the whole box was then clamped together by means of two iron clamps. The vitamin solution was placed in the middle compartment, the two end compartments each being filled with distilled water in which platinum electrodes were immersed. The cathode electrode acted both as stirrer and electrode. A cork pulley coupled to an electric motor was fitted to the upper end of a glass tube, the other end being fused on to the platinum and connection made by means of a copper wire dipping into mercury contained in the tube. This procedure was found necessary as in a previous experiment, using stationary carbon electrodes, a nearly total loss of activity was recorded. This was thought to be due either to adsorption on the carbon or to a local concentration of alkali near the electrode inactivating the vitamin. A cooling coil made of glass tubing was placed in each compartment. The coils were connected in series with a pump and a coil of lead tubing immersed in a freezing mixture, brine being continuously pumped round through this closed circuit to conduct away the heat generated by the passage of the current. During the course of the electrolysis sulphuric acid was added to the cathode compartment and baryta to the anode. The p_H of the middle compartment was similarly adjusted when necessary. In some of the experiments, as indicated below, certain substances were introduced into the end compartments, both to act as buffers and to reduce the resistance of the cell. The p_H was determined colorimetrically by the use of external indicators.

Exp. 1. Electrolysis of lead filtrate at p_H *7 and* p_H *4.*

50 cc. of a lead filtrate fraction prepared by extracting yeast with hot 50 % alcohol, precipitating with lead acetate and removing the lead with hydrogen sulphide were diluted to 150 cc., placed in the middle compartment and adjusted to p_H 7 with baryta. 150 cc. of distilled water were placed in both the anode and cathode chambers. The cathode liquid was kept at p_H 1·5–2, the anode liquid at p_H 2–5. Electrolysis was carried on for 4 hours with a current of 0·8 amp. (the temperature being 10–15°). At the end of this time 15 cc. or 1/10 of the total volume of the middle compartment and

the whole of the anode and cathode solutions were removed for feeding tests. The barium was precipitated by sulphuric acid, the precipitate filtered and washed, and the filtrates and washings were made up to definite volumes. The dosage was computed by finding the number of day-doses present after electrolysis on the basis of the original activity present in the middle compartment. The method of assay of the vitamin was essentially the same as that employed by Guha [1931]. Fig. 1 shows the activity of these solutions and also that

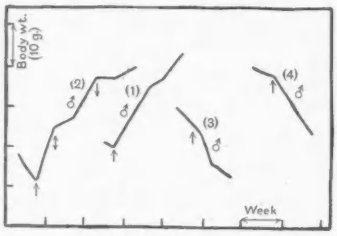

Fig. 1.

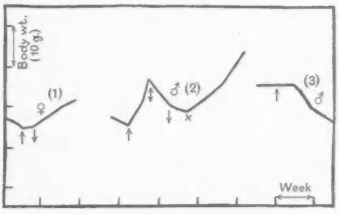

Fig. 2.

Fig. 1.

Curve (1). Lead filtrate before electrolysis; ↑ 0·15 cc. (day-dose).
Curve (2). Fraction of middle chamber after electrolysis at p_H 7·0; ↑ dose≡3 times day-dose of original lead filtrate; ↕ dose≡1·5 times day-dose of original lead filtrate; ↓ dose≡1 original day-dose.
Curve (3). Cathode fraction; ↑ dose≡3 times original day-dose.
Curve (4). Anode fraction; ↑ dose≡6 times original day-dose.

Fig. 2. Electrolysis at p_H 4·4 (Exp. 1).

Curve (1). Middle fraction; ↑ dose≡1·5 times original day-dose; ↓ dose≡4·5 times original day-dose.
Curve (2). Cathode fraction; ↑ dose≡1·8 times original day-dose; ↕ dose≡1 original day-dose; ↓ dose≡1·5 times original day-dose; × dose≡2 times original day-dose.
Curve (3). Anode fraction; ↑ dose≡9 times original day-dose.

of the original lead filtrate. The greater part of the vitamin is seen still to be contained in the middle compartment, the cathode and anode fractions showing no activity. The fluid from the middle compartment was then adjusted to p_H 4·4 and further electrolysed for 4½ hours the current being 0·3 amp. From Fig. 2 it is seen that over 50 % of the activity has passed into the cathode compartment while the middle compartment contains the remainder of the activity, the anode fraction being totally inactive.

Exp. 2. Re-electrolysis of cathode solution at p_H *8·5.*

80 cc. of the active cathode solution obtained at the previous stage were adjusted to p_H 8·5 by means of baryta and re-electrolysed for half an hour with a current of 0·3 amp. at 10–15°. Attempts were made to keep the anode and cathode liquids at approximately p_H 8·5 by buffering them with small amounts, about 0·3 g., of glycine and ammonium sulphate respectively. Owing to the rapid liberation of acid and alkali in these compartments great difficulty was experienced, but for the most part they were kept on the alkaline side of

neutrality. Fig. 3 shows that about 50 % of the vitamin has migrated to the cathode and none to the anode, which confirms the earlier finding.

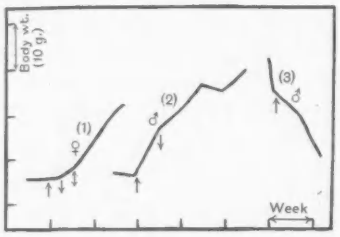

Fig. 3.

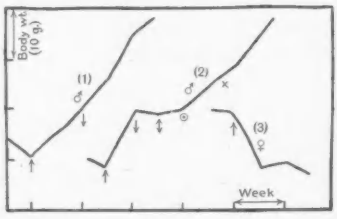

Fig. 4.

Fig. 3. Electrolysis at p_H 8·5 (Exp. 2).

Curve (1). Middle fraction; ↑ dose≡3 times original day-dose; ↓ dose≡4·5 times original day-dose; ↕ dose≡6 times original day-dose.
Curve (2). Cathode fraction; ↑ dose≡8 times original day-dose; ↓ dose≡4 times original day-dose.
Curve (3). Anode fraction; ↑ dose≡12 times original day-dose.

Fig. 4. Electrolysis of a chemically purified fraction at p_H 8·5 (Exp. 3).

Curve (1). Middle fraction; ↑ dose≡2 times original day-dose; ↓ dose≡1·5 times original day-dose.
Curve (2). Cathode fraction; ↑ dose≡1·5 times original day-dose; ↓ dose≡1 original day-dose; ↕ dose≡1·25 times original day-dose; ⊙ dose≡1·5 times original day-dose; × dose≡2 times original day-dose.
Curve (3). Anode fraction; ↑ dose≡4 times original day-dose.

Exp. 3. Electrolysis of a chemically concentrated preparation of vitamin B_1 at p_H 8·5.

This moderately concentrated preparation was obtained from brewer's yeast approximately by the method previously described [Guha, 1931]. The solution was active in a day-dose of 0·25 cc. and contained 1·5 mg. solids (1·3 mg. organic) per day-dose. 21 cc. were taken, adjusted to p_H 8·5, diluted to 50 cc. and electrolysed for 40 mins. with an average current of 0·2 amp. at about 10°. The solutions in the side chambers were treated as in Exp. 2. Fig. 4 shows that nearly 40 % of the vitamin migrated to the cathode under these conditions, which is in agreement with the earlier experiment.

Exp. 4. Electrodialysis of crude yeast extracts at p_H 7 and 4.

A crude yeast extract was made by extracting 1055 g. of yeast with boiling water. The filtrate, 1600 cc., was electrolysed for 12 hours at p_H 7 (1·5 amp.). About 30 % of the vitamin B_1 activity passed to the cathode. The cathode solution contained no vitamin B_2. The solution from the middle compartment was still able to maintain growth of rats totally deficient in the "vitamin B-complex" (Fig. 5). On further electrolysis at p_H 4 there was a loss of vitamin B_2 activity from the middle compartment, with no corresponding increase in activity in either of the side compartments.

Table I summarises the results obtained.

Table I.

Exp.	Conditions	Result	Total organic solids (g.) per day-dose: In the middle chamber before electrolysis	Total organic solids (g.) per day-dose: In the chamber containing the active fraction after electrolysis
1	Lead filtrate electrolysed at p_H 7	Cathode fraction inactive		—
		Anode fraction inactive	0·036	—
		Middle fraction active		0·037
	Further electrolysis at p_H 4·4	Cathode fraction active		0·018
		Middle fraction slightly active	0·037	—
		Anode fraction inactive		—
2	Re-electrolysis at p_H 8·5 of the active cathode fraction obtained in Exp. 1	Anode fraction inactive		—
		Cathode fraction, 50 % activity	0·018	0·024
		Middle fraction, 50 % activity		0·060
3	Chemically concentrated fraction electrolysed at p_H 8·5	Anode fraction inactive		—
		Cathode fraction, 50 % activity	0·0013	0·0012
		Middle fraction, 40 % activity		0·003
4	Electrolysis of crude yeast extract at p_H 7	Anode fraction inactive		—
		Cathode fraction, 30 % activity	—	0·014
		Middle fraction, 60–70 % activity		0·018

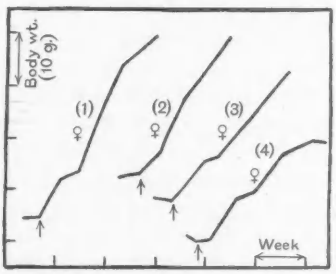

Fig. 5. Electrolysis of crude yeast extract at p_H 7.

Curve (1). Vitamin B_1-deficient rat; cathode fraction; ↑ dose≡3 g. fresh yeast.
Curve (2). Vitamin B_1-deficient rat; middle fraction; ↑ dose≡0·75 g. fresh yeast.
Curve (3). Vitamin B_1-deficient rat; middle fraction; ↑ dose≡3 g. fresh yeast.
Curve (4). Vitamin B complex-deficient rat; middle fraction; ↑ dose≡2 g. fresh yeast; growth falls off later.

Discussion.

From the results of Exps. 2 and 3 it is obvious that vitamin B_1 behaves like a base even at p_H 8·5, and, therefore, must be either a true base or an ampholyte whose isoelectric point is higher than p_H 8·5. Considering the latter case, such an ampholyte might be of the type carrying an excess basic group, or one whose basic dissociation constant is much greater than its acid dissociation constant.

Concerning Exp. 1 with lead filtrate, the fact that the vitamin did not

migrate at p_H 7 and did so at p_H 4·4 can probably be explained by taking into account the resistance of the cell at these p_H values. The much lower resistance at p_H 7 (noted by higher current passing) is no doubt due mainly to the fact that weak acids or ampholytes acting as acids at this p_H value exist as their ionised barium salts so that the barium cations may carry the major part of the current while at p_H 4 they are non-ionised and the barium is precipitated as barium sulphate. Hence the cations at p_H 4 are mostly bases other than barium, and so a much greater proportion of the current is carried by the vitamin. Owing to this variation in resistance of the cell at different p_H values due to the presence of large and varied amounts of ionised impurities, we did not succeed in getting even an approximate idea of the p_K value of the vitamin, since the experimental technique here adopted did not permit of a sufficiently adequate control of all the variables. From experiments with the crude yeast extract it is seen that the vitamin even in the crude state behaves like a base. The fact that the vitamin migrates in the crude state at p_H 7 appears to be in conflict with the result of Exp. 1 but the conditions of the two experiments are not comparable, as the total amount of current passed and the quantity and variety of ionised substances present are not the same. It was found possible to obtain vitamin B_1 in the cathode compartment practically free from vitamin B_2, but it was not possible to obtain vitamin B_2 free from vitamin B_1, as apparently there was a loss of activity of the former during the course of the electrolysis. This loss was probably due to the adsorption of vitamin B_2 on the precipitate of barium salts, proteins, *etc.*, which formed in the middle compartment. Very little of vitamin B_1 is apparently destroyed by electrolysis. Appreciable concentration may be obtained by this method at some of the earlier stages of fractionation.

Summary.

1. Vitamin B_1 in both the crude and relatively purified state behaves as a base even at p_H 8·5.

2. By electrolysis of a crude yeast extract, vitamin B_1 can be concentrated in appreciable quantities free from vitamin B_2 at the cathode.

Our thanks are due to Sir F. G. Hopkins for his interest in this work and to Dr L. J. Harris for valuable suggestions. We wish also to thank Mr A. Ward and Miss R. Leader for the care of the animals. One of us (T. W. B.) is indebted to the Medical Research Council for a grant.

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CLII. BIOCHEMICAL LESIONS IN VITAMIN B DEFICIENCY.

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Several attempts have been made in the past to correlate the symptoms of vitamin B deficiency with failure in the oxidations in the body. In so far as these have been made upon the tissues of polyneuritic pigeons, it is probable though not certain that the work has been actually performed upon the so-called B_1 constituent of the complex. It will not be necessary here to mention more than the salient features of the present position of this knowledge. Those who work with polyneuritic pigeons must soon become aware of the bright red appearance of the blood often present in pigeons showing symptoms. Dutcher [1918] and Findlay [1921] found a fall in catalase and glyoxalase content respectively in certain tissues of the body of pigeons deficient in vitamin B_1—the enzymic power not being restored by the addition of yeast extracts *in vitro*. Hence, vitamin B_1 could not be a co-enzyme for the systems in question. A series of further observations, Abderhalden, with Schmidt [1920], and with Wertheimer [1921] upon the oxygen uptake of tissues, Hess [1921] and Hess and Messerle [1921] upon the reducing properties of tissues for dyestuffs [see also Vasarhelyi, 1926; Roelli, 1923] led to the conception that the fault lay in some failure in tissue oxidations. Some of this earlier evidence does not bear the light of modern criticism; other experimental work has not supported it, in especial the work of Terroine and Roche [1925], of Roche [1925], and of Marrian and Drummond [1926], who obtained no evidence of interference under their conditions either with the oxidations of the animal as a whole, or of the respiration or behaviour *in vitro* to dyestuffs of tissues taken from deficient animals. Though the earlier work is not convincing as it stands, it is not clear that later researches have been performed under precisely the same conditions as those of the original workers. As stated by Gugler [1928], the possible variations in technique are very large. Further it was pointed out by Kinnersley, Peters and Reader [1928] that the symptoms of opisthotonus in the pigeon might be associated with an oxidative deficiency in some small part of the brain, insufficient in weight to be detectable in the oxidation of the tissues as a whole. In this paper it is believed that evidence has been obtained in proof of this opinion. In recent work, Kinnersley and Peters [1930] have found increased lactic acid present, especially in the lower

parts of the brains of pigeons at or approaching the stage of symptoms. This suggested some enzyme deficiency in these parts.

In the following communication it is demonstrated that birds showing symptoms of head retraction, and birds in which these symptoms are threatening, show lowered powers of oxygen uptake *in vitro* in certain parts of the brain, which are marked in the lower parts of the brain. The lowering of oxidation is not general, because the cerebellum shows no such changes. In birds which have been dosed with vitamin, but in which no rise in weight has taken place, the tissue shows a power of oxidation approximating to the normal. This shows that the depression of oxidation found in the avitaminous bird is not merely associated with the state of nutrition as judged by the weight, but is some expression of the avitaminosis.

Experimental.

The oxygen uptake of brain tissue *in vitro* has been investigated by Loebel [1925], Meyerhof and Lohmann [1926], Warburg, Posener and Negelein [1924], Holmes [1930], who have found in rat and frog brain rather large and variable oxygen uptakes, lasting for several hours, which could be maintained at approximately initial intensity for some time by addition of glucose and lactate solutions to the Ringer's solution in which the tissue was suspended. The values obtained by these authors were greater than those recorded by Abderhalden and Schmidt [1920] and Roche [1925]. The latter however worked with the whole brain, mashed and suspended in moist air. It has been shown that white and grey matter have quite different rates of oxygen uptake, Holmes for instance gives 1200 mm.^{3} per g. per hour for brain grey matter, and 300 mm.^{3} per g. per hour for white in presence of air [see also Dickens and Šimer, 1930]. For the purposes of this research there are good reasons for obtaining these measurements with the pigeon, as so much is known about the behaviour of this animal in vitamin B deficiency. It is not however practicable to obtain measurements with the cortex and white matter of the pigeon's brain separately, in fact in the case of most parts of the brain the mixture of grey and white could not be properly separated. Upon this account, our procedure has been to guillotine the pigeons as previously described [Kinnersley and Peters, 1930], remove the two halves of the brain and separate the following parts, the cerebrum, cerebellum, optic lobes and remainder. The tissue has then been finely mashed by the use of small bone spatulas, before transferring to the vessels for analysis. The brain tissue of the pigeon can be rapidly divided in this way, and it is believed to be more satisfactory than other methods for this purpose. If the operations are carried through quickly, little loss of water occurs. After shaking at 38° such brain tissue is found to be finely divided. We have tried to slice as recommended for other tissues, but have not found it satisfactory in the case of the pigeon's brain.

The oxygen uptake[1] was determined in apparatus of the Barcroft type (as modified by Dixon and Elliott [1930]). The corked bottles containing Ringer's solution (3·0 cc.) were weighed before introduction of the tissue and re-weighed after placing the tissue in the fluid, from which the weight of tissue was calculated. The time from the guillotining of the bird to that of placing the apparatus in the thermostat (38° + 0·1) was 12–17 minutes, according to the number of observations in each experiment. The speed of operation was much enhanced by the use of the recent type of air-damped Sartorius balance.

The chemicals used in the research were of A.R. quality. The birds used were Homer pigeons and were fed according to the usual technique employed in this Department. The earlier experiments were done with a Ringer's solution of composition NaCl 0·9 %, KCl 0·025 %, $CaCl_2$ 0·030 %, $NaHCO_3$ 0·015 %. All others with a mixture of 80 parts Ringer with 20 parts $M/2$ acid potassium phosphate, to which was added sufficient NaOH to bring the p_H to 7·4. The precipitate which forms under these conditions was removed before use. It is realised that this mixture may not be ideal for the pigeon, and that research is needed as to the exact composition of the salts for this animal: since all the experiments were strictly comparable, it is not likely that error in the conclusions has been introduced in this manner.

Variations observed and discussion of the errors of the observations.

Despite the trouble which we have taken to work under constant conditions, we have been unable to reduce the occasional variations between duplicate samples of the same normal tissue to less than ± 5 %, though in the majority of cases duplicate observations have shown closer agreement than this. Though we have noticed that the figures of other workers have shown such occasional wide variations, we have tried to discover some of the sources of the variation. For samples of tissue from cerebellum, optic lobe and the rest of the brain, some of the variations are undoubtedly to be ascribed to the difficulty of sampling the mixed tissue: this is not so likely to apply to the cerebrum. Pieces of tissue larger than 0·12 g. tend to give low results, but there appears to be no marked difference in the behaviour of tissue which had been taken from the bird and sampled at room temperature or which had been allowed to cool in ice previously. It is very likely, though not proven here, that many of the variations are due to the failure to control the extent to which the dying cells liberate substrates other than the one studied for the enzyme systems present, or expose enzymes to the action of the substrate studied. As it was clear that the main object of our work was to reveal differences in the behaviour of normal and abnormal tissue, if such existed, we have made no attempt to do more than work under standard conditions, which would make our estimations comparable. In most of the experiments

[1] The constants of the apparatus were determined at approximately 12°, using a modification of Hoffman's method, and corrected for 38° by Warburg's formula. This method was found to be in substantial agreement with direct calibration of the apparatus at 38° by Hoffman's method.

for instance we have worked with oxygen at atmospheric pressure inside the bottles, though we are aware that the oxygen uptake can be increased under our conditions by the use of higher concentrations of oxygen. It is hoped to consider this question later. Meanwhile control experiments have shown us that our conclusions are not influenced by the pressure of oxygen employed. By working with air, we have been able to reduce substantially the time elapsing between death of the animal and the first observations, thereby minimising the possibility of other changes. For a similar reason, no attempt has been made to work with CO_2 present.

The tables show the values obtained in full, and the figures the mean of the recorded estimations, expressed in mm.^{3}/g./hour. The numbers can be readily reduced to Warburg's method of expression [1930] by taking the dry weight as approximately 20 % of the wet weight: *i.e.*

$$1000 \text{ mm.}^3\text{/g./hr.} = Q_{O_2}\ 5;\ 2000 = Q_{O_2}\ 10 \textit{ etc.}$$

Oxygen uptake of pigeon's brain in vitro *in mammalian Ringer's solution without additions.*

In some preliminary experiments performed by one of us in collaboration with Mr R. B. Fisher (April, 1930), the value for the oxygen uptake of normal pigeon's brain tissue in unbuffered mammalian Ringer's solution was found to be in general agreement with those of others. The oxygen uptake was followed for several hours after death and was found to be fairly constant for 30–90 mins., tending to show a slight diminution from 90–120 mins. The values for cerebrum (1000–1380) mm.^{3}/g./hr. were greater than those for the midbrain and optic lobes (1040), and these again greater than for the "rest" (730–930). This difference has been found to persist throughout the experiments. It is not certain that it is due only to the relative proportions of white and grey matter. Comparison of the normal and avitaminous birds revealed little difference between the two, except an indication of a fall for the optic lobes. As the object of our work was to determine the differences between the normal and abnormal, we restricted the subsequent periods of observation to 30–90 minutes after death.

Oxygen uptake of pigeon's brain tissue in vitro *in phosphate-Ringer with addition of glucose (0·25 %).*

In these experiments, the following technique was adopted uniformly. The apparatus containing the samples of tissue was placed in the bath 12–17 minutes after death. It was then shaken for 10 minutes with the taps open, after which the taps were closed. Readings were then made over a period of 60 minutes and the oxygen uptake for the sample considered to be the average uptake of oxygen during the period in question. In the majority of experiments the readings during the hour were consistent; there was a tendency in some experiments with the avitaminous "lower parts" for a slight fall towards

the end of the hour period. Though there was only a small difference between the normal and avitaminous birds for the oxygen uptake in the preliminary experiments, in which Ringer's solution alone was used, the difference is accentuated in the presence of glucose. Table I shows the actual values obtained in the various experiments. In Fig. 1 there is shown upon the chart

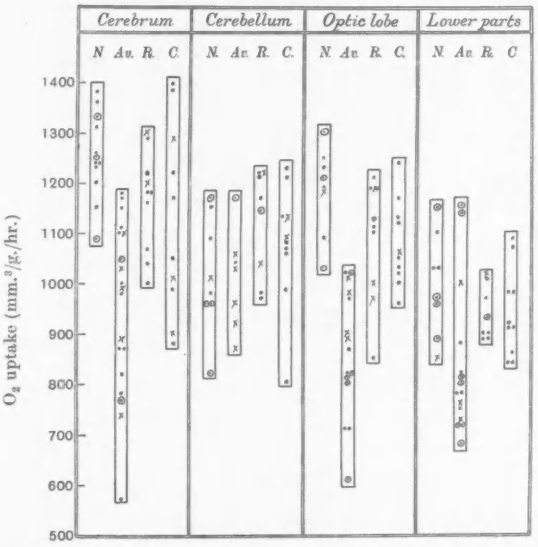

Fig. 1. Oxygen uptake *in vitro* of pigeon's brain (mm.^{3}/g./hr.). *N.* = normal; *Av.* = avitaminous; *R.* = rice fed; *C.* = cured avitaminous. • Average of two estimations agreeing within ±5 %. ⊙ Single determination. × Average of two estimations, not agreeing with ±5 %.

the average figures in the experiments. It will be seen that the values for optic lobe, cerebrum and the lower parts are reduced for the avitaminous birds; for cerebrum and optic lobes by some 20 % and for lower parts by 20 %, on the average. The values are about the same however for cerebellum. Though there appears to be no doubt about the conclusions for optic lobe and cerebrum, it was possible that the value for the lower parts might not have been significant. The matter has been kindly examined for us by Dr R. A. Fisher (Harpenden) by statistical methods. He has tested normal *v.* avitaminous for cerebrum and for lower parts and regards the difference for cerebrum as clearly significant: for lower parts he regards the difference as also significant, there being little probability of getting so large a deviation by chance. The figures prove that there is a difference in certain oxidation systems between normal and avitaminous brains, especially marked in cerebrum and optic lobes, and not present in the cerebellum. Examination of the chart will show that there was a marked tendency for bad duplicates in the samples from avitaminous brain. This must surely be significant and seems

Table I. *Values for oxygen uptake of pigeon's*

N. = normal; *Av.* = avitaminous; *R.* = rice fed; *C.* = cured

Cerebrum				Cerebellum			
N.	*Av.*	*R.*	*C.*	*N.*	*Av.*	*R.*	*C.*
1410 1360 (1)	1000 970 (4)	1260 1330 (6)	1100 930 (53)	1170 (3)	1170 (11)	1210 1240 (15)	1070 1120 (53)
1090 (9)	770 (8)	1200 1170 (15)	980 1000 (52)	820 (9)	1000 740 (14)	1230 1120 (70)	790 810 (57)
1330 (10)	1050 (11)	1030 970 (70)	1030 880 (63)	960 (12)	970 1150 (52)	1140 (73)	1090 1060 (63)
1250 (12)	1110 950 (14)	1270 1140 (73)	910 860 (65)	960 (10)	1030 920 (55)	1340 1100 (83)	1050 1080 (65)
1360 1360 (13)	1170 1180 (27)	1420 1190 (83)	1240 1200 (67)	1090 1100 (13)	990 850 (56)	980 970 (72)	1210 1050 (69)
1250 1220 (16)	870 870 (52)	1120 1020 (72)	1010 1100 (71)	1200 1100 (16)	1120 950 (78)	990 1100 (54)	990 1000 (71)
1300 1320 (62)	790 1010 (52)	1160 1160 (54)	1460 1340 (75)	940 1090 (62)	1150 1020 (80)	990 970 (58)	1160 1100 (75)
1210 1320 (20)	1150 1050 (55)	990 1090 (58)	1190 1050 (76)	990 970 (86)		1210 1240 (15)	1180 980 (76)
1260 1220 (21)	860 890 (56)	1250 1190 (44)	1360 1420 (77)				1200 1220 (77)
1250 1240 (88)	520 500 (61)	1200 1170 (15)	1230 1360 (96)				1250 1220 (96)
1190 1120 (89)	640 850 (64)		1080 1140 (51)				1000 1080 (51)
1180 1220 (90)	1210 990 (66)						
	1140 1170 (78)						
	1050 940 (80)						
	1200 1160 (84)						
	840 810 (87)						
	780 790 (91)						
Average:							
1260	946	1162	1113	1032	1004	1122	1082

to indicate, as do the results for the different parts, an uneven distribution of certain catalysts.

The differences observed between normal and avitaminous brains are not of importance so far as the phenomena of avitaminosis B_1 are concerned, unless it is found that such differences are not merely due to the lowered state of nutrition—we purposely avoid the use of the word inanition. An avitaminous bird under consideration has lost some 40 % of its normal weight, if

pigeon's brain in vitro (*mm.*^{3}/*g./hr.*).

C. = cured avitaminous. Figures in brackets are exp. no.

Optic lobe				Lower parts			
N.	*Av.*	*R.*	*C.*	*N.*	*Av.*	*R.*	*C.*
1300 1270 (1)	710 720 (4)	870 840 (6)	1070 970 (53)	890 (3)	680 (18)	930 (15)	940 895 (53)
1270 1230 (3)	860 890 (8)	1250 1130 (15)	910 1010 (57)	1150 (9)	810 (11)	860 920 (70)	860 820 (57)
1300 (9)	610 (11)	1030 910 (70)	950 1040 (63)	970 (10)	790 740 (14)	1090 950 (73)	970 870 (63)
1210 (10)	820 990 (14)	1090 1110 (73)	990 1130 (65)	960 (12)	880 880 (27)	870 930 (83)	860 820 (65)
1030 (12)	810 830 (27)	1230 1110 (83)	990 1080 (69)	1020 1050 (13)	800 770 (52)	900 900 (72)	1000 970 (69)
1050 1310 (13)	1140 920 (52)	1170 1250 (72)	1190 1060 (71)	1030 (16)	830 690 (55)	910 870 (58)	820 910 (71)
1200 1180 (66)	830 910 (55)	1100 900 (58)	1190 1120 (75)	780 920 (62)	780 690 (56)	940 1000 (54)	1060 910 (75)
1050 1130 (62)	710 710 (56)	1080 1160 (54)	1210 1274 (76)	1090 1110 (86)	720 (61)	990 1040 (44)	1110 1080 (76)
1050 1240 (86) 1140	800 (61)	980 1080 (44)	1470 1100 (77)		720 (64)		1080 1070 (77)
	820 (64)	1250 1130 (15)	1050 1060 (96)		800 (66)		930 890 (96)
	810 (66)		910 1060 (51)		1160 830 (78)		780 860 (51)
	930 1000 (78)				790 780 (80)		
	1030 1010 (80)				1140 (84)		
	1020 (84)						
Average: 1185	864	1083	1083	997	814	940	929

we interpret its normal weight as the maximum weight reached by the bird feeding naturally upon a mixed corn diet. A method of control which has been used with success has been to feed a bird upon yeast extract together with the amount of polished rice consumed daily by a bird feeding naturally upon polished rice alone [Kon and Drummond, 1927]. Under these conditions the curves for weight loss of two such animals run closely parallel. In the circumstances of our experiments it appeared that the best control would be

a bird which had lost some 40 % of its weight, but in which deficiency of vitamin B_1 had been excluded[1]. It has been recently shown [Carter, Kinnersley and Peters, 1930], that birds at the minimum weight reached upon polished rice do not increase in weight when given a daily dose of our charcoal concentrates. We have therefore obtained the oxygen uptake for the different

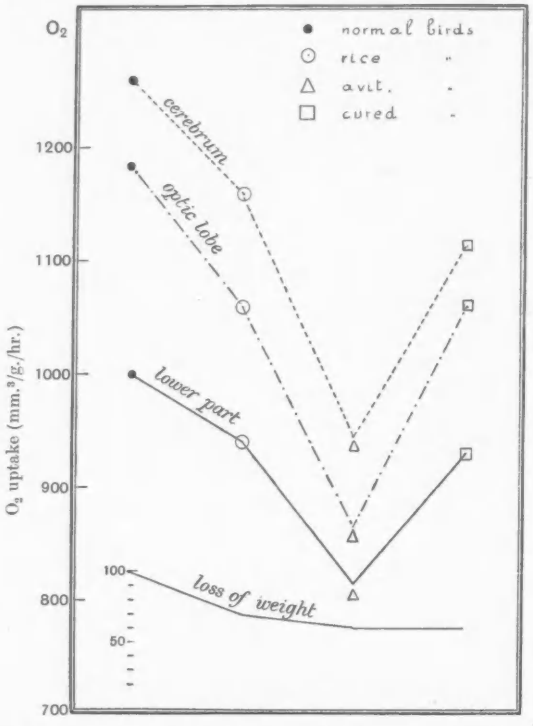

Fig. 2. Diagrammatic comparison of average oxygen uptake of different parts of the brain (pigeon) with the fall in weight at different states of avitaminosis. Ordinates, average oxygen uptake in mm.³/g./hr. Abscissae, periods in the feeding at which the average uptakes were observed. The interval between the first and second points represents 20–30 days; that between third and fourth points 24–72 hours approximately. The diagram includes a curve for the average weight at the same periods, showing that the average oxygen uptakes do not follow the fall in weight in the avitaminous periods.

parts of the brain in (*a*) birds which have been fed upon polished rice until the weight has dropped to some 40 % of the normal, to which doses of vitamin

[1] Throughout this paper we have made the assumption that the changes induced in the oxidations are produced by the vitamin B_1 in the extracts which cures the pigeon. This is justified in the present state of knowledge, because in some cases cures were induced with vitamin B_1 extracts of the *X* type (Appendix) [see Carter, Kinnersley and Peters, 1930]. It should always however be remembered that until a pure preparation of vitamin B_1 is obtainable, any conclusion of this nature can only be provisional.

have been in addition administered (rice in Table I), and (*b*) birds which have been fed upon rice until symptoms appeared and which have then been cured by addition of vitamin (cured in Table I). In some cases the vitamin has been administered for several days before carrying out the experiment.

The results of these experiments are also shown in Table I and Fig. 1, and the averages in Fig. 2. As would be expected the cerebellum shows no change. In the cerebrum, optic lobes and lower parts however dosing with the vitamin has given a rise of approximately 20 % in the rate of oxygen uptake. This proves conclusively that the lowered oxygen uptake is not a question of general inanition but is specifically associated with some entity in the vitamin extracts, which if not identical with vitamin B_1 must accompany it rather closely[1].

In grouping together the results for the avitaminous birds in head retraction and cured birds above, we have purposely overlooked certain minor differences which may influence the values. These are the length of time during which symptoms have persisted, and, in the case of the cured and rice-fed birds, the time elapsing between giving the dose and killing the bird. These points are partly discussed in the Appendix to this paper in which the protocols of the experiments are also given.

In drawing these conclusions as to the significance of the differences between the normal and avitaminous birds, we have considered the possibility that the water content of the brains might be different. This matter was extensively investigated by Roche [1925], who showed only very small differences, not amounting to more than about 2 %. In control experiments of our own we have obtained similar results.

The significance of the changes.

We feel that these results, together with the observations upon lactic acid in the brain [Kinnersley and Peters, 1930], show finally that the symptoms are central in origin in the polyneuritic pigeon. We have evidence of chemical change in the central nervous system on the one hand, and on the other find that the opisthotonus is relieved most quickly by injection of vitamin B_1 concentrates into the pigeon's cranium. From a theoretical point of view the changes might be due either to some general interference with the circulation in the brain, or to actual alterations in the tissue. The fact that the cerebellum does not appear to share in the general fall in oxidations (or in the changes in lactic acid) suggests that we are not dealing with a general failure of circulation. Though the condition of opisthotonus once established is progressive, it is wrong to consider such animals as moribund in the sense that no conclusions drawn at this time can be considered as valid indicators of vitamin B_1

[1] Reference to Fig. 2 shows that the oxygen uptake for the "cured" and "rice-fed" birds dosed with vitamin B_1 concentrates does not rise to the normal value completely. Though this may represent some change, we do not consider that the difference is sufficiently established by our experiments to warrant any far-reaching conclusions.

deficiency. Such birds show such a good blood pressure in many cases that the blood in the severed carotid arteries will spurt for some distance after the use of the guillotine. This leads to the conclusion that the differences in oxidations are not due to the lowered body weight or to failure of circulation. The localised nature of the trouble as well as of the increased lactic acid suggests that the differences are due to lack of some entity in the part affected. Kinnersley and Peters drew the conclusion that there was some change in the tissues of the brain, concomitant with the appearance of the symptoms, which led to accumulation of lactic acid first in the parts of the brain other than cerebrum and cerebellum and gradually involved the cerebrum. This picture seems to be in general true for the oxygen uptake (see Appendix). The other possibility that the depression of oxidation is due to some circulating toxic substance seems unlikely, both because such effects might be expected to be more evenly distributed, and also because, under the conditions of our experiments, the concentration of any such toxic stuffs in the tissues should be rapidly lowered by diffusion into the large volume of Ringer's solution. It seems therefore that the cause of the tissue changes can be one of three possibilities, a lowered content of either (*a*) certain enzymes (oxidases), or (*b*) certain essential substrates other than glucose, or (*c*) certain co-enzymes. The presence of the missing entity is dependent upon the presence of vitamin, as it is restored to the tissue by the cure of the bird. It is hoped that further work will decide whether it is the vitamin itself, or whether the vitamin is an intermediary in the production of some other substance.

Note. In a recent communication Vøgt-Møller [1931] has raised the question whether the symptoms of vitamin B_1 deficiency are due to the accumulation of methylglyoxal in the polyneuritic animal. The evidence for this is based upon the fact that liver extracts from avitaminous animals convert hexosephosphate to methylglyoxal, and not to lactic acid. While this is an interesting observation, in conformity with Findlay [1921], it would be dangerous to believe that lactic acid cannot accumulate in the avitaminous bird, until evidence to the contrary is produced. There is evidence now in the literature for the presence of increased lactic acid in the blood in birds [Collazo and Morelli, 1925; Kinnersley and Peters, 1930] and in man [Hayasaka, 1929], in the brain of the pigeon [Kinnersley and Peters, 1930] and in the tissues of the pigeon [Fisher, 1931]. In view of the possibility that methylglyoxal initially present in a trichloroacetic acid extract might be converted to lactic acid during the process of estimation, we have examined fresh trichloroacetic acid extracts of brain, blood and other tissues of the polyneuritic pigeon for the presence of methylglyoxal by the use of dinitrophenylhydrazine, but have not detected its presence. It can therefore be stated that any amounts of this substance, if present, can only be small; the upper limit would be set by the precipitation reaction with the reagent at about 1 mg. per 100 g. tissue. Since in the work of Kermack, Lambie and Slater [1927] it was found to be necessary to inject amounts of 0·7 g. of methylglyoxal per kg. into animals in order to produce symptoms, it seems unlikely that such small amounts even if present could be responsible for symptoms. As we have evidence of the presence of increased lactic acid, it therefore seems to be unnecessary at present to postulate that the effects are due to some other substance, the presence of which has not yet been demonstrated in the affected part.

SUMMARY.

1. Brain tissue from polyneuritic pigeons (vitamin B_1 deficiency) shows *in vitro* a lowered power of oxygen uptake in the presence of glucose as substrate. This is the case when symptoms are prolonged in all parts of the brain except the cerebellum.

2. Control birds of equal weight to the avitaminous, but previously dosed with vitamin B_1, and birds which have been cured by dosing with vitamin B_1 do not show the same depression of oxidation, which is therefore associated with the specific deficiency.

3. The symptoms of opisthotonus are associated with chemical changes in certain parts of the brain and are central rather than peripheral in origin.

We are indebted to the Government Grant Committee of the Royal Society, and to the Medical Research Council for grants towards expenses, to Dr M. Dixon for valuable help and to Dr R. A. Fisher for aid with the statistical examination of the figures. We are also grateful to Mr R. B. Fisher for the lactic acid estimations.

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APPENDIX.

In Tables II, III, IV are recorded certain of the essential points from the protocols of the experiments, especial attention being drawn to points of interest in connection with the symptoms. In the references to dosing with vitamin B_1, it will be noted that cures have been effected with both the so-called *X* and *Y* preparations [Carter, Kinnersley and Peters, 1930]. The *X* preparations are relatively more free from vitamin B_5 than the *Y* preparations.

Table II. *Cured avitaminous birds* (*C. in Table I*).

Exp. No.	Bird No.	Sex	Days on diet	Loss of weight* %	Date of head retraction 1	Date killed 2	Weight on date 1 g.	Weight on date 2 g.	Lactic acid in blood at death mg./100 cc.	Remarks
51	753	—	22	36	Feb. 10	Feb. 10	172	172	—	Killed 5 hours after vitamin injection. 8 pigeon doses (*Y* preparation). 11.15 a.m., 12.15 p.m., no better. 2 p.m., symptoms cured. 4.15 p.m., practically well except that (1) vision was not completely restored, (2) not able to fly properly
57	456	—	45	47	„ 16	„ 17	280	264	71	7·5 pigeon dose (*X* preparation) injected on Feb. 16. Very fit when killed on Feb. 17
63	701	—	23	37	„ 18	„ 20	240	245	24	Feb. 18 and 19. 3 doses by mouth (*Y* preparation). When killed on 20th still showed weakness in wings, though head retraction completely disappeared on Feb. 19
65	412	♂	35	41	„ 17	„ 23	246	243	45	Feb. 17. 5 doses (*Y* preparation); Feb. 18, 19, 20, 21, 22, 3 doses daily by mouth. Altogether the bird received 24 day doses; bird rapidly recovered from symptoms and seemed well, but even when killed on 23rd, it still showed slight incoordination and difficulty in alighting properly
69	895	♀	22	29	„ 25	„ 26	167	173	34	5 doses (*Y* preparation) given 24 hours previous to death
71	635	♂	39	43	„ 25	„ 27	223	224	26	Feb. 25. 5 doses *Y*, Feb. 26, 3 doses
75	774	♂	37	51	Mar. 2	Mar. 3	258	233	—	Mar. 2. 15 doses (*X* preparation). 3rd flies fairly well. Kept in a dark box for 20 mins. before killing
76	476	♂	28	30	„ 3	„ 4	294	293	—	Mar. 3. 10 doses *X* preparation
77	870	—	32	49	„ 3	„ 4	205	210	—	Mar. 3. 10 doses *X* preparation
96	901	—	28	39	„ 26	„ 27	217	210	—	Mar. 26. 6 doses *X* preparation

* Loss of weight at time of symptoms. In some cases the lactic acid in the blood is not completely normal. This is probably connected with the amount of exercise, though from the results of Fisher [1931] it is likely that the high lactic acid represents a lingering vitamin B deficiency in other parts of the body. The doses were given by mouth, unless otherwise stated.

Note to Table II.

Of the above, the following gave oxygen uptakes below the average for the group of cured avitaminous birds. It is interesting to note that in practically all cases, some abnormality was still noticeable in the behaviour of the bird.

No.	Time after dose	Oxygen uptake: Cerebrum	Oxygen uptake: Optic lobe	Oxygen uptake: Lower part	Remarks
51	5 hours	Average	Low	Low	Vision not restored
57	24 hours	Average	Average	Low	Very fit
63	2 days	Low	Low	Average	Wings still weak
65	6 days	Low	High	Low	Some incoordination still present
71	2 days	Average	High	Low	No observation recorded

Table III. *Avitaminous birds with symptoms (Av. in Table I).*

Exp. No.	Bird No.	Days on diet	Weight loss %	Hours symptoms	Oxygen uptake*			Remarks
					Cerebrum	Optic lobe	Lower parts	
4	339	19	34	6	Average	Low	—	—
14	367	26	41	2	High	Average	Low	—
27	333	18	—	6	High	Average	Average	—
55	644	28	38	—	High	Average	Low	Emprosthotonus and weak
56	632	28	36	30	Low	Low	Low	Emprosthotonus for 30 mins. only. Symptoms threatening for 3 hours previously
74	920	19	34	—	High (1210)	High (1120)	High (1150)	Symptoms threatening only. O_2 uptake, mm.³/g./hr.
84	415	21	30	2	High	High	High	Symptoms threatening only
87	980	24	34	30	Low	—	—	—

* In relation to average for avitaminous birds.

Notes. In this table are included those birds from Table I (avitaminous column) of which the time of onset of symptoms was known. The others were mostly birds which had developed symptoms overnight. In addition, experiment 74 has been added. With exception of 74 and 84, it will be seen that all birds showed a lower oxygen uptake in some part of the brain. In Exps. 14, 27, 55, a lowered oxygen uptake in optic lobe and lower parts was associated with a comparatively normal cerebrum value. This suggests that we are dealing with the same distribution as the lactic acid (Kinnersley and Peters). The lesion seems to commence in the part of brain other than cerebrum-cerebellum, and to extend to the cerebrum as symptoms are continued.

Exp. 74 shows that a bird with threatening symptoms may still show high oxygen uptake. Exp. 84 constitutes an exception in that it showed high oxidation together with symptoms. The interpretation is probably that the bird normally has a higher oxygen uptake than the usual normal.

Table IV. *Rice-fed birds (R. in Table I).*

Mostly dosed in addition with vitamin some hours or days before use.

Exp. No.	Bird No.	Sex	Days on diet	Weight loss %	Date of head retraction 1	Date killed 2	Weight on date 1	Weight on date 2	Lactic acid blood	Remarks
15	447*	—	23	—	—	—	—	—	—	Rice diet (not dosed with vitamin B_1)
44	382	—	30	39	Feb. 4	Feb. 4	290	290	—	Vision good at time of death
53	442	♂	23	32	,, 11	,, 11	265	265	26	10 doses B_1 (*X* preparation) given 90 mins. previous to death
54	732	♂	24	32	,, 11	,, 12	276	275	25	10 doses B_1 (*Y* preparation) on Feb. 11
58	655	♂	29	35	,, 16	,, 17	234	230	36	8 doses B_1 (*Y* preparation) on Feb. 16, 24 hours before death
70	628	♀	38	44	,, 23	,, 27	—	—	—	4 doses B_1 daily Feb. 23–27
72	722	♂	40	44	,, 22	,, 28	272	284	—	4 doses B_1 daily Feb. 24–27
73	814	♀	33	35	,, 22	Mar. 2	214	204	—	Feb. 22, 0·5 g. marmite + 4 doses B_1 (*Y* preparation); Feb. 24–28, 4 doses B_1 daily
83	781	♀	41	40	,, 28	,, 4	283	260	—	Feb. 25, Mar. 3 and 4, 1 g. marmite

* Other data unfortunately missing.

CLIII. CARBOHYDRATE METABOLISM IN BIRDS.

III. THE EFFECTS OF REST AND EXERCISE UPON THE LACTIC ACID CONTENT OF THE ORGANS OF NORMAL AND RICE-FED PIGEONS.

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Kinnersley and Peters [1929, 1930] have demonstrated that there is an increase in the lactic acid content of the brain of the pigeon in polyneuritis (induced by feeding on polished rice) and that the increase is particularly marked in the mid- and hind-brain. They were inclined to interpret their results as an indication that a local defect in the metabolism of this lower part of the brain was responsible for the symptoms of polyneuritis, but it seemed to be of interest to investigate the lactic acid contents of other organs of the body in order to determine whether the phenomenon they observed was in fact a part of a more general disturbance. With this end in view a technique was developed for the use of iodoacetic acid as a "fixative" for lactic acid in the organs of the pigeon, in the hope that it might prove possible to obtain satisfactory estimates of the lactic acid contents of the pectoral muscle, the heart and the liver of the same bird. A preliminary account of this technique has already been published [Fisher, 1931].

In the course of an attempt to obtain standard conditions in the birds used for estimations it was discovered accidentally that the lactic acid contents of organs of polyneuritic pigeons depended to a considerable extent on the conditions of the experiment. This finding has been expanded, and in consequence the results, as presented, are not strictly complementary to those of Kinnersley and Peters. But they serve the original purpose in that they demonstrate that there is a definite general defect in lactic acid metabolism in the polyneuritic pigeon, in addition to the local defect described by Kinnersley and Peters.

Methods.

Lactic acid fixation. The use of iodoacetic acid as a fixative is based on Lundsgaard's [1930] original observation that lactic acid is apparently not formed in the stimulated gastrocnemius of a frog previously poisoned with the drug. The applicability of his assumption to normal pigeon muscle was

[1] Christopher Welch Scholar.

tested by the injection, intraperitoneally and intravenously, into a small series of pigeons of small amounts of sodium iodoacetate. Pectoral muscle from these pigeons was minced and incubated in $M/15$ phosphate buffer, p_H 7, at 37° for 2 hours. In three instances out of four the muscle showed a decrease in lactic acid content, and in the fourth a slight increase was observed. The decrease was not significant unless lactic acid were added to the muscle before incubation.

In view of the possible quantitative importance of the disappearance of lactic acid observed, experiments were made upon the effect of the addition of iodoacetic acid in buffered solution to finely minced unpoisoned muscle. This muscle contained initially from 400 to 550 mg. of lactic acid per 100 g. The rates of glycolysis observed in the presence of these high concentrations of lactic acid ranged from 120 to 610 mg. of lactic acid formed per 100 g. of tissue per hour, at 37°, when the muscle was suspended in buffer in a concentration of 1 g. of muscle to 5 cc. of buffer. But the range of rates of disappearance observed in the same system on the addition of iodoacetic acid was only from 40 to 70 mg./100 g. tissue. The results of these experiments are presented in Fig. 1. It should be noted that control experiments have

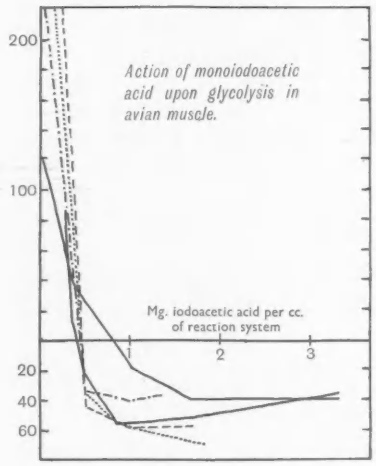

Fig. 1.

shown that no destruction of lactic acid occurs when lactic acid is incubated with iodoacetic acid in the absence of muscle tissue.

Fig. 1 indicates that, in a method of lactic acid estimation by fixation, the error which is produced by lactic acid destruction is likely to be very much less than the corresponding error introduced by glycolysis into the only other method available, *i.e.* rapid excision of tissues from a suitably anaesthetised animal, and immersion of the excised tissues in liquid air. But the figure

understates the case. Two rough experiments in which the iodoacetic acid content of a series of flasks was held constant, whilst the lactic acid content was varied by the addition of *dl*-lactic acid, showed that even at a concentration of 2·5 % total lactic acid (or 1·25 % *d*-lactic acid) the system causing the lactic acid disappearance was not saturated with respect to lactic acid. The rate of disappearance at this concentration was 100 % greater than that at a concentration of 0·5 % *d*-lactic acid. Since the concentration of lactic acid in the tissues taken for lactic acid estimation is in all probability about 0·01–0·02 %, judging by the figures obtained by Davenport and Davenport [1927] for guinea-pig and rat muscle, the rate of disappearance of lactic acid from the tissues under the conditions of *in vivo* fixation by iodoacetic acid should be too small to be of any account.

Since the data of Fig. 1 provide no guide to the best dosage for *in vivo* fixation of lactic acid by iodoacetic acid, the dose had to be found by trial and error. The only criteria available were the uniformity of the results and certain figures which had been obtained in a few preliminary experiments in which I had assisted Professor Peters. In these experiments the pigeons had been anaesthetised with "Liquid Dial," a proprietary non-volatile anaesthetic containing diallylbarbituric acid and urethane, and tissue samples had been removed as rapidly as possible and plunged into liquid air. The range of concentrations of lactic acid found is indicated in Table I. It is of interest to note the extremely high values for the hearts both of the normal and polyneuritic birds. The higher values were the more prevalent in both series, yet all the hearts were beating when they were excised, and it is doubtful if more than 3 secs. ever elapsed between excision and complete freezing. If these figures be compared with those in Table II it will be seen how extraordinarily rapid *post mortem* glycolysis must be in this tissue.

Table I. *Lactic acid content of pigeon tissues; liquid air method.*

Normal	Muscle:	22–35 mg./100 g. tissue
	Heart:	35–94
	Liver:	42–78
Polyneuritic	Muscle:	77–89
	Heart:	63–141
	Liver:	22–96

In all these experiments glycolysis must have occurred to some extent, so that any figure obtained by a successful iodoacetic acid method ought to fall below the lowest figure in the corresponding group in Table I.

Using this criterion it has been found that:

(1) "Dial" anaesthesia militates against success.

(2) Intravenous injection is unsatisfactory.

(3) Fixation is very unreliable in birds which die more than 12 minutes after the injection.

(4) Doses larger than 150 mg./100 g. body weight tend to cause stoppage of the heart before the drug has been adequately distributed.

The method finally adopted was to inject intraperitoneally a dose of 50 or 75 mg. of the drug per 100 g. body weight, the bird being unanaesthetised. The iodoacetic acid was dissolved in 5 % NaOH and made just alkaline to phenol red.

Of the total of 38 sets of estimations reported in this paper 19 were made using a dose of 50 mg./100 g., 18 were made using 75 mg., and one was made using a dose of 100 mg./100 g. of an impure commercial preparation. The higher dosage was used when it became apparent that the figures for the pectoral muscle obtained at the lower dosage were not satisfactory. However, as may be seen from Table II, the increase in dosage had little if any effect on

Table II. *Lactic acid concentrations in pigeon tissues: iodoacetic acid technique.*

1	2	3	4	5	6	7	8	9
		Dose of iodoacetic acid mg./100 g. body-weight	Lactic acid: mg./100 g.			Time to death in mins.	Length of exercise in mins.	Length of rest in mins.
Group	Exp.		Muscle	Heart	Liver			
Rested normals	1	50	6	6	8	4	None	25
	2	50	—	8	14	7	,,	37
	3	50	(39)	6	—	6	,,	37
	4	50	(24)	13	6	12	,,	130
	5	50	(71)	11	21	7	,,	31
	6	50	—	11	19	7	,,	80
	7	75	14	10	8	5	,,	52
	8	75	(25)	—	13	10	,,	45
	9	50	(139)	13	19	7	,,	75
Normals from cage	1	50	11	12	14	—	None	None
	2	50	13	16	15	—	,,	,,
	3	50	(136)	17	10	—	,,	,,
	4	50	12	18	—	4½	,,	,,
	5	50	(28)	13	9	9	,,	,,
	6	50	12	10	7	10	,,	,,
Exercised normal	1	50	15	14	24	12	8	None
	2	75	21	19	18	9	17	,,
	3	75	19	20	32	10	6	,,
	4	75	19	28	54	7	10	,,
Long rest avit.	1	50	—	11	24	7	None	309
	2	50	16	4	19	6	,,	409
	3	75	32	17	20	13	,,	336
Short rest avit.	1	50	41	24	37	6	None	96
	2	50	—	27	—	7	,,	42
	3	50	59	21	20	11	,,	28
	4	75	37	33	35	7	,,	45
	5	75	(133)	27	15	6	,,	26
	6	75	58	26	—	6	,,	150
Exercised avit.	1	75	68	83	70	10	6	None
	2	100	87	76	80	5	5	,,
	3	75	66	51	53	9	11	,,
	4	75	49	64	62	9	10	,,
Cured avit.	1	75	15	18	18	6	—	49
	2	75	13	24	23	6	—	67
Exercised cured avit.	1	75	26	27	37	12	9	None
	2	75	31	20	55	7	16	,,
	3	75	54	33	51	8	11	,,
	4	75	22	26	48	10	12	,,

the nature of the results. The general conclusion which may be drawn from the figures is that the method is eminently satisfactory as far as the heart is concerned, less satisfactory for the liver (though it is conceivable that the range of variation found in the liver corresponds to a true variation of the same magnitude), and of doubtful value for the pectoral muscle. It is interesting to note that the results indicate quite definitely that the drug affects the glycolytic systems of all these tissues.

Lactic acid determination. Samples of the tissues required (one sample from each pectoralis major muscle, the heart, and the greater part of the liver) were removed after the bird had died, pressed between filter-papers to remove adherent blood, and placed in tared 50 cc. centrifuge tubes, each of which contained 10 cc. of 10 % trichloroacetic acid. The tubes were weighed and the tissues were ground up with a glass rod. The tubes were allowed to stand for 45–60 minutes, and the fluid was poured off. The process was repeated three times, using water as the extraction fluid on the last two occasions, and the filtered extracts were collected in 100 cc. flasks. 10 cc. of 10 % $CuSO_4$ solution and 10 cc. of 5 % $Ca(OH)_2$ suspension were added to each flask, and the volume was made up to 100 cc. with washings from the filter.

After standing for an hour the extracts were filtered, and the first 10 cc. of filtrate were rejected. Estimations of the lactic acid content of 10 cc. portions of filtrate were made by the Friedemann, Cotonio and Shaffer [1927] method. *N*/200 iodine, freshly made up, was used for the final titrations, and Na_2HPO_4 was used to liberate the bound bisulphite, as recommended by Lehnartz [1928]. Duplicate titrations agreed as a rule to within 0·02 cc. As the net titration figure ranged from 0·20 cc. to 1·40 cc. the final figures are probably within 10 % of the true values. The method recovers 100–103 % of a standard zinc lactate solution of the order of concentration of the lactic acid in the extracts.

Preliminary treatment of the pigeons.

Polyneuritic pigeons. These birds were obtained by feeding on a polished rice diet and were used in the head-retraction stage. The precautions recommended by Kinnersley, Peters and Reader [1928] to ensure that the head-retraction was truly polyneuritic in origin were observed in each instance.

Cured polyneuritic pigeons. These were birds belonging to the polyneuritic group which had received 1–3 g. of marmite in water by the mouth from 12 to 24 hours before the injection of iodoacetic acid. In all instances the birds were free from any sign of polyneuritis when they were taken, and none of them developed any such sign during exercise.

Rest. The pigeon was kept at rest by wrapping it in a cloth and placing it in a dosing-box.

During the resting period the pigeon was kept in the dark [see, in this connection, Benedict and Riddle, 1929] and at the end of the resting period it was usually possible to take the pigeon out of the box without any move-

ment on its part, provided that a cloth were placed gently over its eyes before it was brought into the light, and it were grasped carefully with the cloth still obscuring its vision. The bird could be turned slowly on to its back and the injection made. It should be noted that the degree of rest is less in the polyneuritic birds than in the normal ones, since the head-retraction persists for some considerable time after they are placed in the dosing-box.

Exercise. It was found to be difficult to ensure that the extent of the exercise given to the birds should be comparable in all the classes (*i.e.* in normal, polyneuritic and cured birds). The normal and cured birds were exercised by being made to fly, either freely, by throwing them into the air, or else by holding them by the feet or tail and repeatedly drawing them downwards through the air. In these circumstances they beat their wings vigorously. The polyneuritic birds were exercised by allowing them free play for their convulsions and by tumbling them about on the ground. In this way normal and cured birds were probably exercised to the same degree, but the severity of the exercise of the polyneuritic birds was definitely less than that of the birds in the two other clases.

Results.

The lactic acid concentrations found in the pectoralis major muscle, the heart muscle and the liver tissue of eight groups of birds are presented in Table II. The figures in brackets in column 4 represent lactic acid concentrations which are in excess of those of the corresponding groups of Table I, *i.e.* results which are obviously not true values. Unfortunately it is impossible, on the data available, to apply the test afforded by Table I to all the groups.

In order that the significance of the variations in lactic acid concentration from group to group may be more readily appreciated, the figures for the lactic acid contents of the heart have been rearranged in Fig. 2 in a different

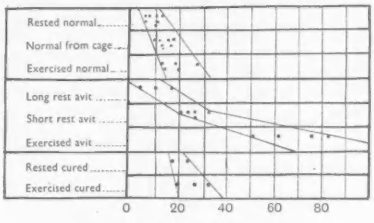

Fig. 2.

form. It has already been said that more reliance is to be placed on these results than on those for the pectoral muscle and liver, but it will be found that what is true for the heart lactic acid content is true also, though not so immediately apparent, for the muscle and liver.

Fig. 2 demonstrates that the effect of severe exercise on the lactic acid content of the heart of the normal pigeon is surprisingly small. Since all the evidence goes to show that in severe exercise lactic acid is liberated in considerable amount, this seems to mean that in the 7–12 minutes which elapse between the end of exercise and the death of the pigeon practically all the liberated lactic acid is converted into some other substance. In the polyneuritic pigeon, on the other hand, we see that the lactic acid content of the heart remains very high for at least 5–10 minutes after exercise, is still twice as high as the normal content in birds from the cage which have been rested for 30–150 minutes, and falls to the level of the resting normal bird only after 300 to 400 minutes' rest.

The only simple interpretation of these results, having regard to their regularity, is that, whereas the normal pigeon possesses a remarkably efficient mechanism for the removal from its tissues of the lactic acid which accumulates in exercise, this mechanism is crippled in great part in birds suffering from avian polyneuritis. This crippling might be an adventitious effect, arising out of the partial starvation which is a consequence of the anorexia accompanying the disorder. Evidence that this is not so is afforded by the observations upon cured birds. There is very little difference between the behaviour of the lactic acid content of the heart in exercise in these birds and in normal birds, whereas there is an immediately apparent difference between its behaviour in cured birds and polyneuritic birds.

As a further proof that what has been termed by some authors "inanition" has nothing to do with the behaviour of the lactic acid content of the heart, the weights of the cured birds at the beginning of rice-feeding, at the time of dosing with the yeast preparation, and at the time of injection with iodoacetic acid, are given for all instances in which accurate data are available. It will be seen (Table III) that the lactic acid content has reverted to normal before any significant rise in weight has occurred.

Table III. *Effect of curative extracts on the weights of polyneuritic pigeons.*

	Weight of bird in g.		
No. of bird in Table II	Before rice-feeding	When dosed	When used
Exercised cured 2	342	230	230
Exercised cured 3	317	192	194
Exercised cured 4	330	251	260
Rested cured 1	365	239	246
Rested cured 2	332	221	228

It seems clear that none of the changes of weight after dosing can be considered to be significant, and that the differences observed between polyneuritic and cured birds must be ascribed to the yeast preparation (in all but one instance marmite) which cured the polyneuritis.

Discussion.

These results are of interest in connection with those obtained by Inawashiro and Hayasaka [1928] and Hayasaka [1929], using Japanese subjects suffering from beriberi. They showed that the blood-lactic acid concentration remained at a high level after severe exercise in beriberi patients for longer than in normal men and cured beriberi patients. They also showed that this tendency to persistence at a high level of the lactic acid of the blood developed progressively in a young male Japanese subject in whom avitaminosis B was induced by feeding him for 200 days on a deficient diet.

Their results conform exactly with the hypothesis suggested to explain the findings reported in this paper.

One point on which some doubt might be felt is the validity of the assumption that while in the normal bird a considerable amount of lactic acid is formed in exercise most of it disappears in the first few minutes of rest. No evidence has been cited in support of this assumption, but Kinnersley and Peters [1930] found 14–34 mg./100 cc. of lactic acid in the blood of pigeons killed with the guillotine (these birds corresponded to the "normals from cage" of this paper), and 80–126 mg./100 cc. in the blood of birds that had been severely exercised (the exercise was in most instances even more severe than that given to the pigeons used in this investigation). These figures are very valuable, since a number of investigations have established that in the absence of substances which inhibit cellular respiration glycolysis does not occur in avian blood. Rüter [1923] established this for goose blood, and Bornstein and Ascher [1926] found the same thing to be true for hen's blood. Gulland and Peters [1930] extended the observations to pigeon's blood. The figures given above may therefore be taken to represent the true lactic acid contents of the blood at the time of death, and it may be considered as established that there is a high concentration of lactic acid in the blood of a normal pigeon at the conclusion of severe exercise. Presumably therefore there must be a concentration of the same order in the organs of the pigeon from which the lactic acid passes into the blood. The fact that Kinnersley and Peters's figures for the blood-lactic acid are on the whole higher than the corresponding figures for the heart (comparing their "normals" with my "normals from cage" and their "exercised" birds with my "exercised avit." birds) can be accounted for if one considers, in the first place, that the exercise they gave their birds was probably more severe, and, in the second place, that the lactic acid of the solid organ is probably not distributed throughout the organ, but is present for the most part in the free water of the tissue. This free water comprises about 77 % of frog's muscle, but no determinations have been made on pigeon tissues [Hill, 1930].

The figures for exercised birds in Table II may therefore safely be considered to demonstrate that a rapid disappearance of accumulated lactic acid takes place in the first 5–12 minutes after the cessation of exercise in a normal

bird, whereas there can be only a very slow disappearance of lactic acid in the polyneuritic bird.

The difficulty encountered by Kinnersley and Peters in relating high lactic acid concentration in the brain to the symptoms of polyneuritis is somewhat diminished on this view. They found that they were able, by exercise, to raise the lactic acid content of the lower parts of the brain in normal birds to the level which they habitually found in the brains of polyneuritic birds. But the normal birds showed no sign of polyneuritis in these circumstances. However, it now seems that the lactic acid content can only momentarily be kept at that height in the normal bird, whereas in the polyneuritic bird it will tend to remain practically stationary for some time. It may be that the persistence of the lactic acid causes an accumulation of some precursor which is responsible for the symptoms. This would fit in with Vogt-Møller's suggestion [1931] that the symptoms of polyneuritis may be due to the accumulation of methylglyoxal, although even Sjollema and Seekles's [1926] comprehensive tabulation of the toxic effects of methylglyoxal does not permit any definite conclusion to be drawn. Peters and Gavrilescu (see note p. 1406) have been unable to isolate methylglyoxal from the brains of polyneuritic pigeons.

Summary.

A method is described for the fixation of the glycolytic systems in the organs of the pigeon by means of iodoacetic acid: the method is satisfactory for the heart, less satisfactory for the liver, and of doubtful value in most circumstances for the pectoral muscle.

Marked differences have been noted between the normal and the polyneuritic pigeon in the responses of the lactic acid contents of the heart, liver and muscle to exericse and rest. It is suggested that these differences signify that in the normal bird lactic acid formed in exercise is very rapidly removed from the tissues, but that in avian polyneuritis the mechanism of removal is very greatly crippled. This crippling appears to bear a close relation to the avitaminosis underlying avian polyneuritis.

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CLIV. THE BIOLOGICAL FILTRATION OF DILUTE SUCROSE SOLUTIONS.

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1. Introduction.

In a recent paper on this subject Jenkins [1931] has shown that in 0·1 % solution either pure sucrose, or mixtures of sucrose and lactic and acetic acids, are readily oxidised on a percolating filter containing $\frac{1}{8}$ to $\frac{1}{4}$ inch clinker to a depth of 54 inches when fed at a suitable rate of flow. The suitability of soluble carbohydrates for the nutrition of micro-organisms needs no emphasis and the more or less complete exhaustion of such solutions is only to be expected provided a sufficient number of the right type of organisms is present under suitable conditions for a sufficient period of time.

The fundamental factors in biological filtration are obviously

(1) the microbial population;

(2) the conditions under which this population exists as determined by

  (*a*) the size of the particles constituting the medium,

  (*b*) the rate of flow of the liquid;

(3) the concentration of the nutrient solution.

The object of these investigations was the more precise determination of the above factors and their application to the treatment of some 250,000 gals. per day of liquor containing 0·2 % of sugar in solution.

The rate of flow used by Jenkins was 9 cc. per minute, equivalent to 86 gals. per cubic yard of filter bed per day. The treatment of 500,000 gals. of a 0·1 % sugar solution at this rate of flow would require a filter bed five-sixths of an acre in area with a depth of 54 inches. It is obviously of some importance to know if these dimensions are the minimum required for the effective solution of the problem.

The complete oxidation of sugar to CO_2 and water requires 1·12 times its weight of oxygen, so that a 0·2 % sugar solution, *i.e.* 200 parts of sugar per 100,000, will require 224 parts of oxygen per 100,000 of water. Since the solubility of oxygen in water is 1 part by weight per 100,000, the solution will require constant aeration during its passage through the filter, and the rate at which this occurs will be a very important controlling factor in the efficiency of the filter. The rate of aeration is determined by the size of particle and the rate of flow of the liquid.

The semi-commercial scale experiments conducted at Colwick, and referred to by Jenkins, showed that the use of fine clinker of $\frac{1}{8}$ to $\frac{1}{4}$ inch was liable to lead to the choking of the filter bed and insufficient aeration, resulting in a very much reduced purification.

Investigations of the inter-relation between concentration of nutrients, the size of particles and rate of flow and their bearing on the rate of oxidation are described in the present paper together with an account of the nitrogen and phosphate requirements of the biological film.

2. The five-day test.

This test measures the polluting value of an effluent by means of the biological oxidation of the organic matter. It therefore achieves the object of the filtration process. For this reason a study of the test will help towards a better understanding of biological filtration.

A sample of the water to be tested is diluted with water saturated with air at atmospheric pressure and 18°. By choosing a suitable degree of dilution the water will supply sufficient oxygen for the oxidation of the organic matter, provided of course that the right organisms are present. Determinations are made by Winkler's method of the dissolved oxygen in the water at the beginning and after 5 days' incubation at 18°, the difference being the amount of oxygen used in the oxidation of the organic matter.

With the object of testing the degree of correlation between sugar content and oxygen absorbed, 5-day tests were carried out on 0·2, 0·15, 0·10 and 0·05 % sugar solutions. The results are given in Table I, dilutions of 1 in 200 being used in each case.

Table I.

Concentration of sucrose solution %	Dissolved oxygen available in parts per 100,000	Dissolved oxygen required in parts per 100,000 (Calculated)	Dissolved oxygen absorbed in parts per 100,000 (Actual)	Sucrose accounted for %
0·20	180	220	131	60
0·15	180	165	102	62
0·10	180	110	62	56
0·05	180	55	35	63

In every case the amount of oxygen absorbed is little more than half the amount required for complete oxidation of the sugar. This discrepancy may be accounted for by (*a*) sugar undecomposed, (*b*) sugar metabolised but not oxidised. To what extent each of these two factors is responsible for the low oxygen absorption would be very difficult to determine. Assuming the unabsorbed oxygen to be due entirely to undecomposed sugar the maximum amount of sugar would be 0·0004 %. Actually most of it will exist as decomposition products of sugar and as cell tissue of the organisms effecting the decomposition. A more extended incubation period is obviously necessary for more complete oxidation. At the extreme dilutions of sugar used in this test the generation time of the organisms must be very considerable, probably 12 hours

or more (see p. 1432). Another series of tests was carried out in duplicate with the same dilution using a 0·15 % sugar solution. Ten bottles were incubated in each case and the temperature of incubation raised from 18° to 25° during the first 5 days. Determinations of oxygen absorbed were made at intervals during 20 days. The results are given in Table II.

Table II.

	Present at start	Dissolved oxygen in parts per 100,000 after 1	2	3	4	5	6	7	10	20 days
I	142	92	55	35	27	24	24	22	19	2
II	142	94	56	35	26	24	24	23	19	2

These results show that the absorption of oxygen is most rapid during the first 3 days and that 70 % of the sugar is oxidised after 4 days' incubation. This result is considerably higher than that obtained in the previous experiment.

Cook and Stephenson [1928] using pure cultures of *B. coli* measured the oxygen absorption in a Barcroft apparatus and found that for glucose only two-thirds of the theoretical amount was absorbed by the organism. They offer no explanation to account for the incomplete oxidation of the glucose and do not consider that bacterial growth may be responsible. In the 5-day test, however, there is an increase in bacterial numbers during the first 2 days and the writer is of opinion that bacterial growth and synthesis is responsible for the incomplete oxygen uptake. The differential rates of oxygen uptake as well as the different amounts recorded by Cook and Stephenson [1928] for various carbohydrates seem to point to a difference in value of these carbohydrates in metabolic synthesis (see p. 1431). This subject is being investigated in greater detail.

The determination of the possible variation due to the inoculation was made by preparing a series of tests using tap water only for dilutions, and another series with the addition before dilution of 1 % of a laboratory filter effluent to the sugar solution to be tested. The results are given in Table III.

Table III.

No. of sample		Oxygen absorbed per 100,000	Standard deviation
1	Tap water only	91	Mean = 92 ± 7·4
2		87	
3		89	
4		108	
5		87	
6		89	
7	Tap water *plus* 1 % filter effluent	112	Mean = 110 ± 3·1
8		109	
9		115	
10		107	
11		113	
12		106	

These results show that leaving the dilution water to a chance inoculation causes a decreased oxygen absorption and a greater variation in the amount than using a definite inoculation from a filter bed.

The standard deviation of the mean using a definite inoculation is 3·1 which means that a single determination may vary as much as 9·0 % from the mean value.

As an accurate method of determining the amount of organic matter present in a liquid this test is obviously not very reliable. A controlled inoculation is not practicable since there is no known method of producing cultures of bacteria in a uniform state of efficiency.

The possibilities of a chemical test answering the purpose are not encouraging. Recent work at Rothamsted has shown the 4-hour permanganate test to be unsuitable for estimating the oxygen requirement of organic acids [Water Pollution R.B. Report, 1929]. The only reliable method is that of combustion, and this could not be considered as a true measure of the polluting effect, since there are organic substances which are comparatively inert biologically. There appears therefore to be no alternative to this incubation test, and, provided too much reliance is not placed on single determinations, it may be considered to answer the purpose for which it was designed. In the succeeding experiments it has been used as a measure of the amount of purification effected by biological oxidation on percolating filters. In this case the calculations are made from determinations obtained by using identical inoculations.

3. The biochemical oxidation of sucrose.

A very extensive literature exists on this subject which for the purposes of this paper may be summarised as follows.

After hydrolysis of the sucrose the hexose sugars give rise to methylglyoxal ($CH_3.CO.CHO$) which yields lactic acid ($CH_3.CHOH.COOH$) and then by oxidation pyruvic acid ($CH_3.CO.COOH$). Pyruvic acid on hydrolysis yields acetic and formic acids. Formic acid is readily decarboxylated yielding CO_2 and H_2. Alternatively pyruvic acid may be decarboxylated yielding acetaldehyde which on reduction yields alcohol and on oxidation acetic acid. Acetaldehyde may also form the starting-point of several organic syntheses by polymerisation and condensation forming higher fatty acids and alcohols.

In biological filtration any or all of these compounds may be produced according to the bacterial population and conditions of aeration of the filter bed. In any case the fundamental processes in all the different types of fermentation are the same, and the object of filtration, *i.e.* the complete oxidation of the carbohydrates to CO_2 and water, is best secured by reducing the synthetic reactions to a minimum. In accordance with Hopkins's dictum the production and accumulation of any particular compound in the filter bed, other than the final products of oxidation, is a measure of its uselessness to the organisms producing it [Stephenson, 1930]. From the point of view of

pollution the intermediate products, lactic and acetic acids, theoretically have the same oxygen absorption value as sugar, so that their production does not result in any reduction in the polluting effect of the liquid. Their decomposition requires the presence of hydrogen acceptors which may be provided either by free oxygen or oxygen combined as nitrate [Quastel, Stephenson and Whetham, 1925]. Since the solubility of oxygen in water is very slight, it may become the limiting factor determining the rate of oxidation. It has long been known that denitrification is part of the respiratory process of bacteria under conditions of restricted aeration. The presence of oxidised nitrogen either as nitrites or nitrates in a filter bed is generally considered as indicating its efficient working, but the possibility of their playing a part in the actual oxidation of carbohydrates does not appear to have been previously recognised.

4. Experiments with sectional filters.

With the object of obtaining more information as to the working of a biological filter, a special filter was designed by the writer and manufactured in earthenware by Messrs Doulton of Lambeth. The principle of this filter was adopted by Jenkins and described in a recent paper [1931]. The original apparatus consists of pipe sections each 1 foot in length and 4 inches in diameter fitted at one end with a perforated plate containing 5 holes $\frac{1}{2}$ inch in diameter. Each section was set on a separate earthenware saucer provided with a single drainage hole 1 inch in diameter. The object of the saucer was to collect the effluent from each section and deliver it to the centre of the next section below. In this way the possibility of the liquid passing down the inner surface of the pipes was obviated. A certain amount of spreading of the effluent over the surface of each section was effected by the presence of a ring of drip nipples round the lower edge of the drainage hole of each saucer.

The capacity of each section, assuming a 9 inch working depth, was such that when supplied with 1 litre of liquid per 24 hours it was equivalent to a filter bed receiving 100 gallons per cubic yard per day (g.y.d.). Five such sections placed in a column can be conveniently supplied with 5 litres of liquid per day from a 6-litre aspirator at this unit rate of feed. The possible criticism that such a filter would be subject to abnormal conditions on account of the supposed increased aeration due to the air gaps between each section is not valid. The contraction of the film in passing from one section to another would result in less aeration than if the filter medium were continuous. It should be remembered, however, that the choking of any one section not only affects that particular section, but in practice would also put out of action the whole column.

In filtration experiments the rate of flow of the liquid through the bed is obviously of great importance and precautions must be taken to ensure that the rate of flow is actually what was intended. In these experiments the aspirators were fitted with a Mariotte's constant head device which released

the liquid under a very low uniform hydrostatic pressure. The use of a "T" piece outlet obviated the possibility of gravitational pull by the dropping liquid and at the same time facilitated cleaning operations. The apparatus was kept free from both organic and mineral deposit by rinsing with hydrochloric acid each day. Before sampling, checks on the rate of flow were made by measuring the volume of effluent collected in 30 minutes.

A. *The size and nature of the filter medium.*

In this experiment two columns each of six sections were used. One column was filled with fine gravel of $\frac{1}{4}$ to $\frac{3}{8}$ inch and the other with clinker of $\frac{3}{8}$ to $\frac{5}{8}$ inch. Each column was inoculated with 100 cc. of a suspension of film obtained from a large scale experimental filter being operated at a Sugar Beet Factory. Both laboratory filters were fed with a solution of the following composition at the basic rate of 100 g.y.d.

			%
Sucrose	...	...	0·2
NaCl	...	...	0·03
K_2HPO_4	...	...	0·03
KH_2PO_4	...	...	0·02
$MgSO_4$	...	...	0·01
$(NH_4)_2SO_4$	...	...	0·03
$CaCO_3$	...	...	0·03 (supplied by the tap water).

The p_H was 6·8–7·0.

This experiment was run continuously for a period of 60 days. Samples of the effluents of each section were examined daily for p_H and mineral nitrogen and weekly for degree of purification by means of the 5-day test.

(*a*) p_H *values*. These were determined by means of the B.D.H. capillators, with bromocresol purple and bromothymol blue. The results were plotted as curves with the numbers of the sections as ordinates and the p_H values as abscissae. During the first week little or no change in the reaction of the liquid occurred in its passage down the filters so that the graph is almost a straight line. During the second week the p_H of the effluents from the second section became more acid (see Figs. 1 and 2).

By the end of the second week acidity developed in the effluent from the first section and continued to increase during the third and fourth weeks, but to a greater extent in the effluent from the gravel filter than in that from the clinker. This difference in acidity between the two filters continued throughout the remainder of the experiment. The p_H values of sections 1 and 2 of the gravel filter averaged 5·5 whilst in the corresponding clinker sections the average was 6·0. The effluents were always less acid from section 3 and generally reached neutrality in section 4. In sections 5 and 6 they became slightly more alkaline (p_H 7·0–7·1) than the original solution supplied from the aspirator. This development of alkalinity in the lower sections diminished after the 30th day with the increase in nitrification, more especially in the case of the

clinker filter in which the effluent gradually became more acid (p_H 6·5) after leaving section 4 (see Fig. 2).

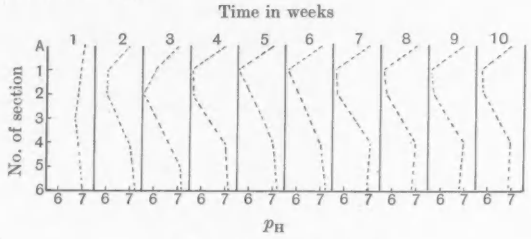

Fig. 1. p_H of gravel filter.

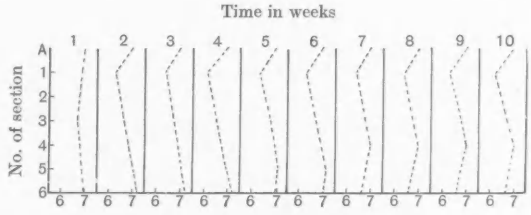

Fig. 2. p_H of clinker filter.

The greater acidity developed in the gravel filter may be due to deficient aeration, unless some basic material in the clinker was exerting a neutralising effect.

An attempt was made to diminish the acidity of the gravel filter by placing glass dishes filled with broken chalk beneath the drainage holes of sections 1 and 2. Although the chalk was at first rapidly corroded by the acid liquor there was no significant change in the reaction of the effluents. After two days the chalk was covered with a gelatinous film which prevented any further corrosion. On testing the liquid in the dishes containing the chalk it was found that the p_H was even less than that of the liquid dropping on to it from the section above. The same lowering of the p_H also occurred on collecting the effluent in glass dishes without any chalk. In 2 hours the p_H dropped from 5·5 to 5·0 and in 20 hours 4·2 was recorded. The liquid at the surface gave a p_H value of 5·8. This higher p_H of the surface layer could only be accounted for by greater aeration.

In order to confirm the improbability of any buffering effect of the clinker medium the basicity of the media was determined by titration with both hydrochloric acid and acetic acid.

With both mineral and organic acids the gravel contains more basic material than the clinker. The difference between the two is greater in the case of the organic acid on account of the occurrence of sulphides in the clinker which are not decomposed by acetic acid.

Time of shaking (hours)	Gravel	Clinker
	cc. of $N/10$ HCl acid neutralised	
24	7·3	5·8
48	9·0	8·8
72	9·4	9·15
	(Some H_2S was evolved by the clinker.)	
	cc. of $N/5$ acetic acid neutralised	
24	3·7	0·5
48	8·0	0·6
72	8·6	0·7

It must therefore be concluded that the difference in reaction between the two filters is due entirely to aeration and not to chemical composition. Jenkins [1931] states that "for acid to be produced the p_H of the nutrient solution must not be less than 6·7 to 6·8." It is difficult to reconcile this statement with the above observations or with any known theory of fermentation.

The cause of this difference in aeration must be attributed to the size of the particles of gravel. Although the total volume of interstitial space is independent of the size of particles, the permeability to water is proportional to the square of their diameter [Green and Ampt, 1912]. With small particles the interstitial space is also diminished to a greater extent by the film of liquid and bacterial slime. After running for 45 days the first section of the gravel filter was completely blocked with film and the liquid ran over the side of the section. This filter was therefore considered to be out of action since the lower portions only worked in virtue of their being sectional. The clinker filter continued working in a satisfactory manner for another month.

The gravel filter was therefore dismantled and the amount of film present in each section was determined in the following way.

(*b*) *Determination of weight of film in gravel filter.* The gravel from each section was shaken thoroughly with successive lots of 400 cc. of water and the total volume of suspended film made up to 2 litres. This was allowed to settle and 1 litre of clear water was decanted. The remaining litre of film suspension was then thoroughly agitated and 60 cc. of it were evaporated to dryness, dried at 100° and weighed again. It was then ignited and the ash determined.

The results are as follows.

Table IV.

Section	Total weight of film g.	Weight of ash g.	Weight of volatile matter g.	Ash %
1	14·6	4·8	9·8	33
2	14·0	6·2	7·8	44
3	11·4	6·2	5·2	54
4	9·4	5·2	4·2	55
5	12·6	7·2	5·4	57
6	5·2	3·4	1·8	65

These results show

(1) that the greatest amount of film occurs in the first section and that

the amount generally diminishes with depth; the irregularity of the fifth section is probably due to collection of film discharged from higher sections;

(2) that the ash generally increases in percentage amount with depth and constitutes the bulk of the film in the lower sections.

(*c*) *Reconstructed gravel filter.* This filter was then reconstructed with coarse gravel as follows:

Section 1, 2	½ to ¾ inch	Coarse.
,, 3	⅜ to ½ inch	Intermediate.
,, 4, 5, 6	¼ to ⅜ inch	Fine as before.

It was then inoculated with the wash water from the previous medium.

As in the previous experiment acidity first developed in section 3 at the end of the first week, after which it occurred only in the first (p_H 5·6) and second sections (p_H 6·2). After the second week the p_H of the first section varied between 6·0 and 6·5 whilst that of the second section rarely fell below 6·5. The p_H curves obtained with this graded gravel were almost identical with those obtained with the clinker filter, thus confirming the conclusion that for the above experiment the size of the particles and not their chemical composition or texture determines the development and efficiency of the film.

Both filters continued to operate in a very satisfactory manner for 20 weeks when both were choked in the lower sections by copious discharge of film from the sections above.

(*d*) *Purification.* The purification effected by a filter is calculated from the oxygen absorption figures in the 5-day test of the original solution and of the effluent. The difference between these two determinations expressed as a percentage of the first is the percentage purification. The results obtained during the first period of the two filters are given in Table V.

Table V.

Section	Percentage purification 1st	2nd	3rd*	4th	5th	6th	7th	8th	week
				Gravel filter.					
1	42	43	16	21	22	21	3·3	15	
2	58	77	27	46	40	55	23	40	
3	55	90	43	78	66	68	41	32	
4	68	92	57	86	87	82	66	77	
5	68	90	69	96	95	84	85	87	
6	72	90	76	93	96·5	84·5	89	94	
				Clinker filter.					
1	27	52	42	60	22	38	34	36	
2	30	74	59	78	40	46	55	66	
3	52	76	68	90	54	65	80	93	
4	58	76	73	93	81	77	90	98	
5	72	86	81	96	90	85	90	100	
6	73	90	79	97	91	93	95	100	

* Nitrogen omitted.

These results lead to the following conclusions.

(1) There was no difference between the filters during the first 3 weeks. Both gravel and clinker media gave 90 % purification after 2 weeks.

(2) Omission of nitrogen and consequent retardation of the growth of film lowered the efficiency immediately.

(3) Most of the purification was effected in the first three sections and very little effect was produced by the sixth section (see Fig. 4).

(4) The fine gravel filter showed a fall in efficiency in the first section after the second week, but without any appreciable effect on the lower sections.

The changes in the purification of each section are shown in Figs. 3 and 4.

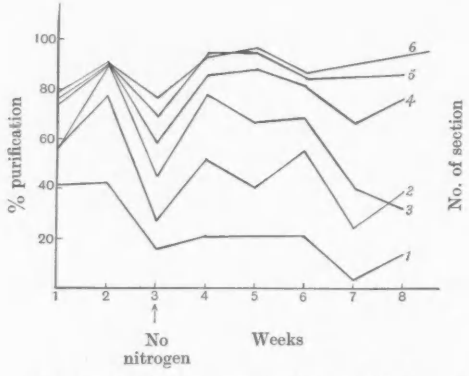

Fig. 3. Purification curves for fine gravel filter.

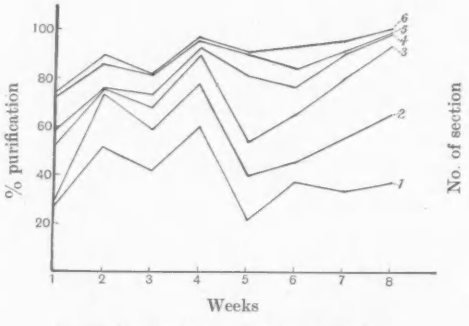

Fig. 4. Purification curves for clinker filter.

The purification results of the second period after reconstructing the gravel filter are as follows (Tables VI and VII).

These results show:

(1) that the coarse gravel ($\frac{1}{2}$ to $\frac{3}{4}$ inch) was much superior to fine gravel ($\frac{1}{4}$ to $\frac{3}{8}$ inch) and to clinker ($\frac{3}{8}$ to $\frac{5}{8}$ inch); the purifications in the first two

Table VI. *Coarse gravel (regraded).*

	Percentage purification							
Section	1st	2nd	4th	6th	9th	11th	13th	15th week
1	26	64	61	77	68	20*	46	69
2	45	88	74	86	85	73	69	90
3	60	90	84	91	86	85	82	92
4	75	94	93	95	89	95	92	95
5	83	94	95	95	96	95	95	95
6	85	95	94	95	96	95	97	97

* Drainage outlet closed by pebble.

Table VII. *Clinker.*

	Percentage purification					
Section	1st	3rd	5th	9th	12th	14th week
1	22	26	22	20	45	32
2	64	69	33	35	55	43*
3	80	85	59	40*	67	72
4	86	95	87	90	92	90
5	95	97	90	95	96	95
6	97	97	95	99	98	98

* Choked with larvae and debris, and cleared.

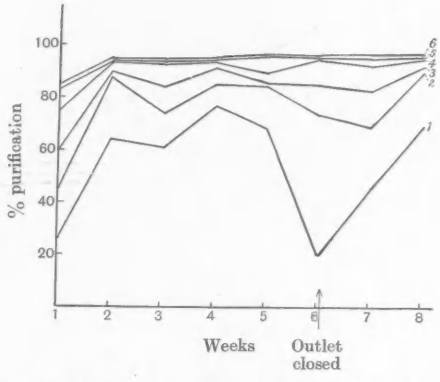

Fig. 5. Purification curves of regraded (coarse) gravel filter.

sections were much higher and showed less falling off in efficiency with age (Fig. 5);

(2) that starting the filter with the previous inoculation showed more rapid development of purification than with the original film from the large scale filter bed;

(3) that 3 feet of gravel ($\frac{1}{2}$ to $\frac{3}{4}$ inch) will give 90 % purification of 0·2 % sucrose solution after 2 weeks and maintain this for more than 16 weeks;

(4) that very little extra purification was obtained after the 4th section in either filter.

B. *Oxidation of lactic and acetic acids.*

The difference in acidity and purification between the coarse and fine filter media suggested a possible correlation between p_H and purification. An experiment was therefore carried out using 0·2 % solutions of lactic and acetic acids in place of sugar. The experiment ran for 14 days and was characterised by remarkably good purification figures and nitrification.

The results are given in Tables VIII and IX.

Table VIII. *Oxidation of lactic acid.*

			Purification %		
	p_H	$[H^·] \times 10^7$	2nd day	7th day	14th day
Aspirator	3·7	2000	—	—	—
Section 1	4·8	158	16·6	43	58
,, 2	6·5	3·2	63·0	83	83
,, 3	6·9	1·1	85·0	91	99
,, 4	7·0	1·0	90·0	98	100
,, 5	7·1	0·8	93·0	99	100
,, 6	6·9	1·1	96·0	99	100

Table IX. *Oxidation of acetic acid.*

			Purification %				
			2nd day	7th day	14th day	21st day	
			0·2 % acetic acid			0·2 % sucrose	
	p_H	$[H^·] \times 10^7$					p_H
Aspirator	4·0	1000	—	—	—	—	6·9
Section 1	4·6	250	40	36	38	44	6·6
,, 2	6·5	3·2	65	82	85	57	6·8
,, 3	6·8	1·6	87	94	97	78	6·9
,, 4	7·0	1·0	90	96	98	91	7·0
,, 5	7·1	0·8	93	96	98	98	6·9
,, 6	7·0	1·0	95	98	98	98	6·9

The reduction in $[H^·]$ is seen to be much greater in the first section than in any of the other subsequent sections, but the amount of purification effected is about equal in the first and second sections. Obviously the reduction in acidity is effected by other processes in addition to oxidation. The results show very conclusively that the development of acidity in a filter bed is not in itself detrimental to its efficiency. If anything, it appears that organic acids are more readily oxidised than sugar, a result which is not in agreement with conclusions reached by Jenkins [1931]. It follows therefore that the low purification of the fine gravel filter is not due to any possible toxic action of the organic acids, but solely to lack of oxygen. These results are also of great interest biologically since the protozoan population of sections 1 and 2 was almost entirely extinguished, so that the more efficient filtration process was carried on by bacteria and fungi supporting a population consisting of eelworms and insect larvae.

The more rapid oxidation of organic acids compared with that of sugar may have a possible explanation in terms of energy transformation.

$$C_6H_{12}O_6 = 2C_3H_6O_3 + 22{\cdot}5 \text{ kg. cals.}$$
(Glucose) (Lactic acid)

$$C_6H_{12}O_6 = 3C_2H_4O_2 + 45 \text{ kg. cals.}$$
(Acetic acid)

$$C_3H_6O_3 + 3O_2 = 3CO_2 + 3H_2O + 326 \text{ kg. cals.}$$

$$C_2H_4O_2 + 2O_2 = 2CO_2 + 2H_2O + 209 \text{ kg. cals.}$$

In the fermentation of sugar to lactic and acetic acids the organisms may derive energy for growth and metabolism without oxidising the material, but no growth is possible on lactic and acetic acids without oxidation. When sugar is supplied to a filter bed there is doubtless a development of organisms capable only of utilising intra-molecular energy, and these organisms must necessarily die out when the filter receives only organic acid, resulting in an increase in the oxidising organisms.

C. *The development of the film.*

The active portion of the filter medium consists of bacteria and fungi supporting a very variable population of protozoa, nematodes and insect larvae. It would be difficult to follow the possible fluctuations in the bacterial flora but they are doubtless reflected in the constantly changing fauna. In a filter bed the organisms develop in the form of colonies attached to the particles of the medium. On starting up a newly inoculated filter the liquid shows an increasing bacterial population as it flows through successive sections, until a certain maximum is reached about the third section, when clumping or colonisation begins, resulting in a much reduced dispersion of the bacteria in the lower sections. This process continues and gradually appears in the upper sections until all the effluents show a marked reduction in bacterial dispersion. The effect of this is shown in Table X by the results of direct chamber counts of bacteria in the effluents.

Table X.

Section	Bacterial numbers in millions per cc.	
	1st day	14th day
1	5·6	32–52
2	32	20–45
3	296	14–29
4	84	12–13
5	36	10–16
6	36	8–14

(These figures do not represent in any way the bacterial population of the film but only the free organisms in suspension. The numbers in the film vary from 3000 to 9000 millions per g.) As the fixed colonies proliferate they may become too heavy for the point of attachment and thus break away either as a whole or in portions. These small fragments of film are carried along with the liquid, provided the filter bed is coarse enough to allow them to pass through, and they may be seen in the effluent from all sections after the first or second week of operation. Under the microscope they appear as masses

of micrococci in the form of zoogloea with occasional yeast cells and funga hyphae. Frequently they carry with them large numbers of protozoa and eelworms. The movements of the larger eelworms and annelids may also play a part in the breaking away of the colony. The colour of the film is at first white especially in the upper sections, but after 3 weeks it changes gradually to a dark brown in the lower sections. This shedding of film is a constant process in the life of the filter and is quite distinct from the periodical sloughing which is associated with seasonal climatic changes.

The rate of growth of the film will be greatest in the first section where the concentration of food is greatest and will diminish with the depth of the section. This explains the greater purification effected in the upper sections of the filter bed. According to Penfold and Norris [1912] the time of generation of *B. typhosus* in peptone increases from 60 minutes at 0·2 % concentration to 90 minutes at 0·1 % and 180 minutes at 0·05 %. A similar result was obtained by Cutler and Crump [1923] for protozoa, the concentration of the nutrient in this case being expressed in terms of bacterial numbers. With a purification of 75 % in section 3 the concentration of nutrient in this section would correspond to 0·05 % sugar and, assuming the time of generation in mixed culture to be similar to that in pure cultures, it would be about three times that of the first section, so that the film would only grow at one-third the rate. Similarly the growth rate in the fourth, fifth and sixth sections would be reduced almost to nil. The figures given in Table IV however show much greater amount of film than would be expected from these growth rates. This difference from expectation must be attributed to the film continuously shed from the upper sections being retained by the fine gravel in the lower sections. The use of a coarse medium would allow an easier passage to this discharged film. The effect of this is seen in the reconstructed gravel filter. When the first three sections were filled with coarse gravel the discharge of film into the surface of section 4 (fine gravel) was so great that it became covered in a period of 12 weeks with a solid layer $\frac{1}{2}$ inch thick.

D. *Time of contact.*

The development of the film has an effect on the filter apart from its increase in population, *viz.* in increasing the "time of contact" or the length of time the liquid takes to pass through the filter. This factor is usually determined by Clifford's method [1907] which measures the mean time required to wash out a quantity of sodium chloride from the filter at a given rate of flow.

It is obvious that the time of contact will increase with the development of the film. The film consists of about 97 % by weight of water and the total volume in one of these experimental filters may be $1\frac{1}{2}$ litres. With a rate of flow of 5 litres per 24 hours the water of the film can be changed $5/\frac{3}{2}$ times in 24 hours which gives a time of 7·2 hours for each change of water.

The volume of film in each section is measured by the difference between

the volume of the interstitial space of the clean gravel, and that after development of the film. This determination was carried out on one of the sectional filters filled with gravel of $\frac{1}{2}$ to $\frac{3}{4}$ inch after running 1 month. The results are given in Table XI. The time of contact is the ratio $\frac{\text{volume of film}}{\text{rate of flow}}$, since if X = volume of film and V = total volume of liquid per 24 hours, then V/X = number of times the film is filled per day, and $\frac{24}{V/X}$ = time of contact = $X\,24/V$.

Table XI.

Section	Volume of film cc.	Rate of flow	Time of contact hrs.
1	450	5 litres per day	2·1
2	400		2·0
3	300		1·5
4	150		0·7
5	75		0·36
6	75		0·36

From the above table we find that out of a total of 7 hours of contact 5·6 hours are spent in the first three sections. The time of contact is greatest where nutrition and growth are greatest. By reducing the size of the gravel, thereby causing a retention of the film, the time of contact can be increased considerably but not without diminishing the aeration, and increased contact without aeration is of no value. Increase in the time of contact should be the result of growth rather than mechanical accumulation. A deficiency in time of contact only occurs when starting up a new filter, and an excess tends to occur with age. This initial deficiency can be best remedied by increasing the concentration of the nutrient solution thereby increasing the rate of growth of the film. If at the same time the rate of flow is diminished, the capacity of the filter will not be exceeded. As the film develops the concentration of the solution can be diminished and the rate of flow increased.

E. *Rate of flow.*

It has been shown that the development of film in a filter is greatest in the upper sections and that it is determined by the concentration of nutrients in the liquid. Increase in the rate of flow will diminish the time of contact. This will result in a lower purification gradient, which means a higher concentration of nutrient in the lower sections, and an increase in the development of film below the upper layer. Thus with a lower purification gradient a deeper filter becomes necessary.

A problem of long standing to the sewage engineer is the question whether it is better to filter a given amount of organic matter as a strong solution at a slow rate or a dilute solution at a quicker rate. Information on this point can be obtained from a consideration of the previous figures for the sectional purification. In Table VI by averaging the figures for the normal working periods, the 2nd, 4th, 6th, 9th, 13th and 15th week, we get the following percentage purification: section 1, 64 %; section 2, 82 %; section 3, 88 %.

Since the rate of flow through five sections is 100 g.y.d. the rate of flow through each section alone is five times as much or 500 g.y.d. A 0·2 % sucrose solution run at this rate undergoes a purification of 64 %, whilst the effluent of this section containing the equivalent of 0·072 % sucrose, run through the second section at the same rate, undergoes a purification of 18 % of 0·2 = 0·036 or 50 % and the effluent of section 2 containing the equivalent of 0·036 % sucrose undergoes a purification of only 6 % of 0·2 = 0·012 or 33 %.

Thus a reduction in the concentration of nutrient through a filter diminishes its efficiency, though the purity of the final effluent is increased. The effect of increasing the rate of flow with a reduction in concentration of sucrose was studied by means of two sectional filters filled with the same gravel, $\frac{1}{2}$ to $\frac{3}{4}$ inch. Both were inoculated with the same medium. One was run with a 0·2 % solution of sugar at 100 g.y.d. and the other with 0·1 % solution at 200 g.y.d. The experiment was run for 30 days after which the gravel was washed and the amount of film determined in each section.

The results of the purification are given in Table XII.

Table XII.

	Number of days							
	6		13		20		30*	
Section	0·2 %	0·1 %	0·2 %	0·1 %	0·2 %	0·1 %	0·2 %	0·1 %
	Purification %							
3	90	59	90	72	88	74	72	50
6	96	90	99	98	99	97	97	83

* Nitrogen omitted from both filters.

There was little difference between the final purification in both filters until nitrogen was omitted from the solution. But throughout the experiment the purification at section 3 was distinctly in favour of the more concentrated solution. The effect of dilution is not apparent with a filter 6 feet deep but with a shallow filter of only 3 feet the use of a more dilute liquid at increased rate of flow gives a lower purification.

The weights of film on the various sections are given in Table XIII.

Table XIII. *Distribution of film with different concentrations of sucrose.*

	0·2 % solution at 100 g.y.d.		0·1 % solution at 200 g.y.d.	
Section	Total film (g.)	% ash	Total film (g.)	% ash
1	16·5	26	15·8	26
2	16·0	32	14·9	33
3	10·4	37	12·5	38
4	6·0	42	7·8	44
5	4·3	44	5·6	57
6	4·1	46	4·5	59
Total	57·3		61·1	

It would seem that the amount of film is greater in the upper sections and diminishes more rapidly with the 0·2 % solution at 100 g.y.d. than with the

0·1 % solution at 200 g.y.d. With the more rapid rate of flow the amount of film is greater in the lower sections, part of this increase being due to a greater ash content.

F. *The influence of the C/N ratio on the film.*

The effect of omitting nitrogen from the sugar solution has been shown in the purification figures in Tables V and XII. Most of the previous work on biological filtration has been done with liquids of a high nitrogen content and having a C/N ratio less than 10. The pulp-press liquor from a beet sugar factory has a C/N ratio of about 20, but since most of the nitrogen exists as organic nitrogen it is not immediately available for the nutrition of the organisms of the filter. Such a liquor would thus resemble a liquid with a higher C/N ratio. The growth of the film in the filter bed has a certain definite nitrogen requirement without which its development will be retarded. An analysis of the film from the top and bottom sections of the gravel filter (see Table IV), is given in Table XIV.

Table XIV. *Nitrogen content of film.*

Section	Total nitrogen (Kjeldahl)	% organic matter	% nitrogen in organic matter
1	7·1	67·0	10·6 = 69 % protein
6	4·6	38·7	13·1 = 85 % protein

The percentage of nitrogen in the organic matter is high, especially in the film of section 6 which contains nitrifying organisms. It is equivalent to a protein content of 85 % so that the organisms of the lowest section have a very low non-protein content (15 %) similar to that of animals in a state of starvation. Probably they are living on their own tissues since there is practically no soluble organic matter in the liquid passing through this portion of the filter.

A C/N ratio of 20 is equivalent to a nitrogen content of 2·1 % of the weight of sugar. In order to increase this nitrogen content to that of the film in the upper section, *viz.* 10·6 %, 4/5 of the sugar must be oxidised to CO_2. The remaining 1/5 of the sugar is then just sufficient to combine with the 2·1 % of nitrogen to form the organic matter of the film. If more than 4/5 of the sugar were oxidised there would not be sufficient to combine with all the nitrogen, and the excess nitrogen would be excreted. Thus, the nitrogen requirements of the film can be measured by adding the nitrogen as ammonium sulphate and noting its rate of absorption in the various sections of the filter.

For this purpose four filters were run on 0·2 % sugar solutions containing different amounts of ammonium sulphate, so as to give C/N ratios of 5, 10, 15 and 20 respectively. The effluents from the various sections were tested for ammonia with Nessler's reagent. A large excess of ammonia occurred in all sections of the filters with ratios of 5 and 10. In the filter with a ratio of 15 the excess ammonia was very slight in sections 5 and 6, whereas with a ratio of 20 no ammonia was found in sections 4, 5 and 6. It appears therefore that the limit of the nitrogen requirements of the filter medium lies between the

ratios of 15 and 20, which is in agreement with the oxidation of 85 % of the sugar and the conversion of 15 % into organic matter of the film.

To test the possibility of nitrogen passing through the filter as amino-acid, formaldehyde titrations were carried out on the effluent from sections 2 and 3. The results are given in the following table.

Table XV. *Formaldehyde titration values of filter effluents.*

	C/N ratio			
Sections	5	10	15	20
	cc. of *N*/10 NaOH required per 100 cc. of effluent			
2	4·3	1·8	1·1	0·1
3	3·9	1·7	1·0	0·1

The titration value is negligible except in the case of a C/N ratio of 5. A titration value of 4 cc. is equivalent to 0·0056 % amino-nitrogen or 1/3 of the nitrogen supplied as ammonium sulphate. It must be concluded that amino-nitrogen is not produced in excess of the requirements of the organisms except in the case of a low C/N ratio. Similar determinations on the effluents of the fine gravel filter showed an increase in the formaldehyde titration value from 2·0 to 3·7 when ponding occurred, which suggests that the formation of amino-acid in the filters was due to some disturbing factors: in the one case possibly excess of NH_4 ions (ammonium sulphate), and in the other lack of aeration. The absence of ammonia from the effluents of the filter with a C/N ratio of 20 can therefore only be due to nitrogen shortage.

The percentage purification results obtained with the various C/N ratios are given in Table XVI.

Table XVI. *Percentage purification.*

		C/N ratios			
Days	Section	5	10	15	20
2	6	83	86	79	78
7	6	93	91	92	83
14	1	41	36	30	32
	2	68	60	71	58
	3	91	73	88	76
	4	94	91	89	84
	5	95	92	90	90
	6	95	92	92	92
21	1	58	59	56	53
	2	74	76	75	72
	3	95	82	85	86
	4	98	97	94	92
	5	99	98	97	96
	6	99	98	98	98
28	1	18	28	45	43
	2	42	57	51	61
	3	60	67	83	73
	4	81	71	87	94
	5	91	90	90	96
	6	97	96	96	97

It will be seen that in the early stage of the development of the film a low C/N ratio gives a better purification, but this difference does not persist beyond 14 days. On the 21st day there is practically no difference between the various filters as regards purification in any of the sections. On the 28th day the filters with the low C/N ratio show a distinct falling off in purification in the first three sections, but there is no significant difference between the final effluents.

The filters were dismantled after 30 days and the amounts of film on each section determined. The results are given in Table XVII.

Table XVII. *Effect of C/N ratio on dry matter of film.*

	C/N ratio							
	5		10		15		20	
Section	Film	Ash	Film	Ash	Film	Ash	Film	Ash
	g.	%	g.	%	g.	%	g.	%
1	18·7	21	18·6	20	16·8	25	17·1	22
2	17·9	25	17·7	27	16·4	31	15·3	27
3	12·8	33	11·8	34	10·4	37	9·7	37
4	9·7	40	9·7	40	6·4	42	7·0	43
5	4·8	56	5·0	62	4·3	44	6·6	62
6	4·1	59	4·2	63	4·1	51	4·0	63
Total	72·3		71·0		58·4		63·3	

Total weight of sugar consumed per filter = 300 g.

Deducting the ash content of the film we obtain a total weight of film produced of 50·0 g. from the C/N 5 filter and 39·2 g. from the C/N 15 filter, representing 17 % and 13 % respectively of the weight of sugar consumed. This leaves 83 % and 87 % to be accounted for by the sugar oxidised and that remaining as impurity in the final effluent. These results are rather better than those obtained in the 5-day test (p. 1421), but inferior to the 90 % obtained by Cutler and Crump [1923] in the pure cultures with continuous aeration.

From these results it would appear that the better purification obtained in the early stages with a low C/N ratio (5–10) is due to a more rapid growth of film, which may later become excessive and diminish aeration with consequent reduction of efficiency.

Some control over the growth of film would appear to be desirable for the maintenance of efficiency. Of the various limiting factors to bacterial growth, two only appear capable of application in practice, *viz.* (1) dilution, (2) reduction of nitrogen (only applicable where nitrogen has been added). Direct biological control of the population has not yet been attempted, but experiments in this direction would doubtless supply useful information.

A reduction in growth rate of the organisms can be effected by diminution in the nitrogen supply to a C/N ratio of 100. This occurred when ammonium sulphate was omitted from the solution, but in such cases there was an immediate drop in the purification. It must therefore be concluded that a certain growth rate is essential for efficient filtration.

The maintenance of mature organisms with a high CO_2 output does not appear a possible solution. The most hopeful line of biological control of the film would appear to be in the use of a metazoan population to effect a more rapid consumption and discharge of film (*cf.* acetic acid experiment, p. 1430).

G. *Nitrogen fixation.*

Microscopic examination of the effluents of the various sections revealed the presence of organisms resembling the capsular and sarcina forms of *Azotobacter*, the nitrogen-fixing organism. Various workers on filtration have suggested the possibility of nitrogen fixation taking part in the processes of the filter bed. A sectional filter was therefore run for 14 days on 0·2 % sugar solution *plus* mineral salts but without added nitrogen. The tap water used for dilution contained 8 parts of nitrate-nitrogen per million, equivalent to a C/N ratio of 100. The gravel was washed before commencing the trial and a small inoculation of the previous film was used. To assist the establishment of a film 0·006 % nitrogen as ammonium sulphate was added to the first day's charge in the aspirator.

The purification figures and weights of film obtained are given in Tables XVIII and XIX.

Table XVIII. *Purification of nitrogen-free solution.*

Section	Percentage purification	
	After 4 days	After 14 days
3	52	58
6	65·5	76

Table XIX. *Weights of film from nitrogen-free solution.*

Section	Weight in g.		Total nitrogen %
	Total	% ash	
1	17·6	8·1	
2	10·1	11·0	2·02
3	12·1	14·5	
4	7·7	21·0	
5	6·9	34·0	1·46
6	5·9	35·0	

Total nitrogen of the film (Table XIX):

$$\left.\begin{aligned} &\text{From sections 1, 2 and 3} \quad 39{\cdot}8 \times \frac{2{\cdot}02}{100} = 0{\cdot}8039 \\ &\text{From sections 4, 5 and 6} \quad 20{\cdot}5 \times \frac{1{\cdot}46}{100} = 0{\cdot}2993 \end{aligned}\right\} = 1{\cdot}1032.$$

Nitrogen supplied in solution:

$$\left.\begin{aligned} &5 \text{ litres} \times 0{\cdot}00006 \text{ g. N per cc.} = 0{\cdot}3 \text{ g.} \\ &13 \times 5 \text{ litres} \times 0{\cdot}000008 \text{ g.} \quad = 0{\cdot}52 \text{ g.} \end{aligned}\right\} = 0{\cdot}82$$

Gain in nitrogen = 0·2832 g.

Sugar passed through filter

$$14 \times 5 \text{ litres} \times 0{\cdot}002 = 140 \text{ g.}$$

Assuming 70 % consumption of sugar by the organisms

Sugar consumed = 98 g.

Ratio of sugar consumed/nitrogen fixed $= \frac{98}{0{\cdot}2832} = 345.$

These results show a small but definite gain in nitrogen similar in amount to that obtained by various workers on nitrogen fixation. The effluent contained no mineral nitrogen but probably contained a small amount of organic nitrogen in the form of living organisms, so that the total fixation of nitrogen was no doubt greater than the above figures show.

From the point of view of biological filtration nitrogen fixation cannot be said to be of any real importance. With a C/N ratio of 100 the amount of nitrogen fixed is only adequate to affect a purification of 76 % after 2 weeks' running with a 6 ft. filter. Assuming the same increase in purification with depth as in previous experiments it would require a filter at least 12 ft. deep to effect a purification of 98 % using a solution with a C/N ratio of 100.

H. *Nitrification.*

The production of nitrates in a filter effluent has always been a criterion of purification. In the experiments with sectional filters the effluents from each section were tested daily for nitrite by the Griess-Ilosvay reagent. In starting up a new filter with 0·2 % sugar a period of 20 days may elapse before any nitrite appears in the effluent. The effluents from the solutions of C/N ratio 5 and 10 showed the presence of nitrite several days before the effluent from a C/N ratio of 15 or 20, and the amount of nitrite was always greater from the solution of low C/N ratio.

The amount of nitrite produced diminishes from section 6 upwards to section 4, but none is found in the upper sections irrespective of the C/N ratio. The presence of nitrifying organisms in these upper sections has been shown by applying the Griess-Ilosvay test to the dilution water after incubation in the 5-day test. These sections therefore provide an example of the suppression of nitrification even with a C/N ratio of less than 5. It would appear therefore that the degree of purification has a greater influence on nitrite formation than has the C/N ratio [*cf.* Jensen, 1929]. When this figure reaches 85 to 90 %, *i.e.* equivalent to not more than 0·03 % sugar, nitrification may appear. It has been shown that bacterial growth is much more rapid in the upper sections, being 6 times more rapid in section 1 than in section 4. This growth would have a large oxygen requirement which would be met by the reduction of any available reducible substance. In these circumstances nitrous oxide could not persist. In sections 4, 5 and 6 aeration is generally more than sufficient to maintain the oxygen requirements of the organisms. For these reasons nitrite formation only persists during the final stages of purification.

The presence of oxidisable carbohydrates does not in itself necessarily inhibit nitrification. Nelson [1931] has recently shown that *Nitrosomonas* in

pure culture may continue to oxidise ammonia even in the presence of 10 % glucose.

In the case of the filters run on lactic and acetic acids the rate of purification was more rapid and strong nitrification appeared in section 3.

Certain pure strains of bacteria isolated from the filter bed at Colwick appeared to be capable of oxidising ammonium lactate to CO_2 and nitrous acid simultaneously, but in the presence of restricted aeration the nitrite did not persist [Rothamsted, 1929].

In the case of the clinker filter supplied with a solution of C/N ratio 15, the amount of nitrite in the final effluent reached 3 parts per million on the 30th day, and increased to a maximum of 35 parts on the 50th day. With the increase in nitrification all trace of ammonia disappears from these sections. The maximum amount of nitrite-nitrogen obtained in this experiment is slightly more than half the total amount supplied to the filter as ammonia. This means that nearly half the nitrogen added with the sugar is retained in the filter as organic matter and is only slowly released by decomposition.

Considerable fluctuations in the amount of nitrite were recorded from time to time, but they were generally traceable to irregularities in the rate of flow or mechanical stoppages resulting in deficient aeration. In the fine gravel filter nitrification steadily declined after the 30th day as a result of the diminishing purification of this filter. After the regrading of the upper three sections nitrification reappeared in the lower sections and continued vigorously.

The production of nitrates was detected 10 days after the appearance of nitrites, but it developed more slowly, apparently requiring the stimulus of larger amounts of nitrite. Increase in the amount of nitrate occurred at the expense of the nitrite, until all trace of nitrite disappeared from sections 5 and 6 and only a small amount remained in section 4. The amounts of nitrate in the final effluent varied considerably from time to time from 1 part N to as much as 10 parts N per 100,000, which is more than the rate of supply of nitrogen to the filter. To some extent these variations were due to differences in aeration but not entirely. The fact that they were of a much higher order than occurred in the previous period of nitrite formation suggests that the variation is of a biological nature. Fluctuations in the C/N ratio of the original solution had no direct effect on the amounts of nitrate in the effluent.

To obtain further information on this point, section 6 of the filter was removed and treated as a separate filter. Solutions containing nitrogen in different forms and amounts were run through this section at the rate of 2 litres per day (Table XX). The solution of each substance was run through the filter for not less than 24 hours. Estimations of the three forms of mineral nitrogen were made by the colorimetric methods as described in the Ministry of Health Bulletin [1929], ammonia by Nessler's reagent, nitrite by Griess-Ilosvay reagent, and nitrate by the pyrogallol test.

Table XX.

All quantities expressed in parts per million of solution.

Source of nitrogen	Total N	Amount of N in effluent as			Gain or loss	
		Ammonia	Nitrite	Nitrate		
Ammonium lactate	200	40	4	120	—	−36
,,	150	100	8	44	+ 2	—
Nil. Water only	—	—	0·5	40	+ 40	—
,,	—	—	1·0	24	+ 25	—
Ammonium lactate	60	40	1·0	4	—	−15
,,	60	30	0·2	32	+ 2	—
Urea	30	1	0·2	16	—	−13
,,	60	10	0·2	13	—	−37
Nil. Water only	—	—	—	12	+ 12	—
Ammonium sulphate	140	40	1	100	+ 1	—
,,	60	50	—	16	+ 6	—
Sodium nitrite	40	—	15	40	+ 15	—
,,	20	—	0·2	40	+ 20	—
,,	60	—	18	120	+ 78	—
Nil. Water only	—	—	—	20	+ 20	—
Sodium nitrite	60	—	8	80	+ 28	—
,,	100	—	15	120	+ 35	—
,,	100	—	0·2	200	+100	—
,,	100	—	10	160	+ 70	—
,,	100	—	0·2	160	+ 60	—
,,	180	—	48	320	+188	—
Nil. Water only	—	—	—	32	+ 32	—
,,	—	—	—	24	+ 24	—
Sodium nitrite	100	—	16	160	+ 76	—

From these results it appears that the only nitrogenous substance capable of stimulating nitrate formation is sodium nitrite, which also gives rise to more nitrate-nitrogen than is supplied as nitrite. The increased nitrogen excreted in presence of nitrite is more than can be accounted for by the normal oxidation of the nitrogen in the film, since water alone extracts much smaller amounts of nitrate-nitrogen. It is suggested that the toxicity of the nitrite exerts some selective action on the mixed bacterial flora of the filter, resulting in increased breakdown and oxidation of the film. The symbiosis between these ammonifying and nitrifying bacteria is the subject of detailed bacteriological investigation.

J. *Phosphate metabolism of the film.*

In the preceding experiments the mineral salts added to the sugar solution included 0·05 % potassium phosphate (p. 1424). The amounts of available phosphoric acid in each effluent of the sections was estimated by the colorimetric method of Fiske and Subbarrow [1925].

The results are plotted in curve A, Fig. 6, the amounts of phosphoric acid found in the various effluents being expressed as percentages of that supplied in the sugar solution.

As would be expected the absorption of phosphate is confined to the first three sections or the region of growth of the filters. In the 6th section there is a slight increase in the amount of soluble phosphate in the effluent probably due to the oxidation of organic phosphorus and the nitrification process.

These changes in phosphate content of the effluents may be correlated with changes in the p_H of the sectional effluents. In Figs. 1 and 2 it has been shown that the p_H of sections 4 and 5 tends to increase above that of the original solution, whilst in section 6 it tends to become less. The absorption of phosphoric acid by the growing film in sections 1 to 3 would result in the formation of free potash which would combine with any organic acid produced by the decomposition of the sugar. Oxidation of these salts would then result in the formation of potassium carbonate and a consequent rise in p_H. In section 6 the oxidation of organic phosphorus and nitrification would result in a lower p_H in this section.

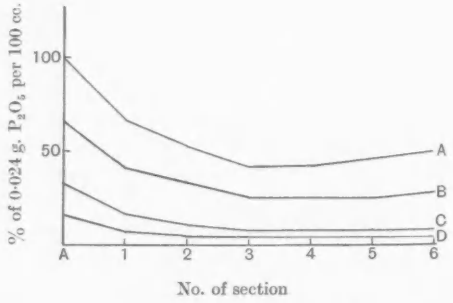

Fig. 6. Absorption curves for phosphoric acid in sectional filter.

Attempts to measure the phosphate requirements of the film by the method employed in the case of nitrogen were not successful. Reducing the amount of phosphate in the original solution even to 1/6 the amount did not result in complete absorption of the phosphate by the film (see Curves B, C and D, Fig. 6). Each reduction in phosphate supply merely diminished the phosphate gradient down the various sections. This phenomenon is probably due to the division of the phosphate between the demand for nutrition or tissue formation and the temporary requirement of the enzyme reactions involving the formation of hexosephosphate. The hydrolysis of the hexosephosphate by the mineral acid used in the colorimetric determination would account for the apparent presence of uncombined phosphate during phosphate starvation.

The effect of phosphate deficiency on the working of the filter was determined as follows.

The five sectional filters were washed and re-inoculated with equal amounts of wash water. They were then run for 3 weeks on a basic solution of 0·2 % sugar and 0·03 % ammonium sulphate to which was added potassium phosphate in different amounts as shown in Table XXI.

The highest purification figures were given by filter C receiving 0·008 % P_2O_5. Increasing the amount of phosphoric acid did not improve the filtration

Table XXI. *Percentage purification at section 3 with different amounts of phosphates in the crude liquor.*

Filter ...	A	B	C	D	E
P_2O_5 % ...	Nil*	0·004	0·008	0·016	0·024
Days of run					
2	55	57	68	64	64
8	60	65	75	73	75
15	54	74	95	95	90
21	51	75	74	72	70

* The tap water used in this experiment contained 0·08 part P_2O_5 per million.

though Fig. 6 shows that it results in an increased retention of phosphoric acid by the film. This probably indicates a greater growth of film and since it is not accompanied by a corresponding increase in purification it must be assumed that aeration has become the limiting factor. This would explain the falling off in efficiency after the 15th day.

A smaller amount of phosphoric acid than 0·008 % appears to delay the development of the film, and in the almost complete absence of phosphate the film is permanently reduced in oxidising power.

Conclusions.

Biological filtration consists of two processes, *viz.* (1) deposition of organic matter by growth of film; and (2) oxidation through respiration. Both processes must be provided for, and since they are dependent upon one another the dominating factor in a filter bed is aeration, which is determined initially by the interstitial space and subsequently by the growth of the film. In this connection it should be realised that the minimum size of the particles is the determining factor and not the average size.

The growth of film is an essential part of the process in the filter, and any condition which delays or prevents it has an adverse effect on the purification. The most important factors influencing this growth are the concentration of food material or organic matter in the solution and its composition. Both these factors are more prominent in the initial stages of the working of the filter and their effect diminishes with age. Similarly their effect is greatest in the upper layers of the bed and diminishes with depth. An adequate supply of nitrogen and phosphorus is essential to the growth of the film and when the C/N ratio is more than 15 and the C/P_2O_5 ratio more than 10 the development and efficiency of the film is reduced. The lower portion of the filter bed then becomes dependent on nitrogen fixation and the discharge of organic nitrogen and phosphorus from the upper layer. In the early stages both these sources of nitrogen are inadequate and consequently delayed purification results. This effect of a high C/N ratio is probably one of the chief difficulties in the treatment of sugar beet factory effluents.

Physiologically a satisfactory filter bed is characterised by the absence of putrefaction (*i.e.* the production and accumulation of readily decomposed

organic compounds). (See Table XV.) The energy of decomposition is consumed in growth so that the occurrence of dead organisms is only a momentary phase in the metabolism of the film. Considering the enormous protozoan population, the occurrence of dead organisms is very rare. Under anaerobic conditions however decomposition products may accumulate and result in a change in the p_H of the medium and a higher formaldehyde titration value.

The clumping and colonisation of the organisms in the bed of the filter is also a very characteristic feature. It results in a much lower bacterial population in dispersion through the solutions than would normally occur in a liquid culture. This clumping or agglutinating process appears to be connected with the degree of aeration. The effluents from the fine gravel filter were generally opalescent and passed through a filter paper with difficulty, whilst those from the coarse filters were clear and filtered through paper readily. The degree of opalescence diminishes with the depth of the filters.

Nitrification is an essential feature of an efficient filter bed since oxidised nitrogen provides the most effective hydrogen acceptor in biological oxidation.

Nitrite formation occurs when the concentration of sucrose and its derivatives in solution falls below 0·03 %. The absence of nitrification in the presence of a higher concentration of soluble organic matter does not imply the absence of the nitrifying organisms but rather that the potential rate of decomposition of the nitrite is more rapid than its production. The production of nitrite ultimately gives rise to the production of nitrate, and this process increases until nitrite formation is hardly detectable.

Summary.

1. There are two limitations to the 5-day oxygen absorption test, *viz.* (*a*) it is not possible to estimate carbohydrate in terms of oxygen absorption by a biological process owing to organic synthesis being an essential part of the process; (*b*) the nature of the inoculum affects the relative amounts of oxidation and synthesis.

Recognising the above limitations, the 5-day test is the most satisfactory measure of pollution available.

2. The production of organic acids is a stage in the decomposition of sugar, but their rapid destruction is a property of an efficient filter bed.

3. The organic acids lactic and acetic are more rapidly oxidised than sucrose.

4. The formation of oxidised nitrogen in the presence of organic matter promotes the dehydrogenation (oxidation) of lactic acid and probably of other compounds.

5. The growth of film is an essential part of the purification process and the film itself accounts for 15 % of the sucrose supplied.

6. Aeration is the dominant factor in biological filtration and is determined by the minimum size of the material which provides the interstitial

space for the growth of the film and also for its discharge. A fine medium should not be used to secure time of contact.

7. If grading of the medium is desired, the smaller particles should be placed at the bottom where the smallest amount of film will develop, but to ensure free passage of discharged film nothing passing through $\frac{1}{2}$ inch mesh should be used.

8. The rate of flow is the best means of controlling the time of contact and purification. Within limits a slow rate of flow with high concentration will increase the time of contact more rapidly than the reverse conditions. As the time of contact increases, the rate of flow may be increased without reduction in purification.

9. The growth and efficiency of film is dependent upon definite nitrogen and phosphorus requirements, *viz.* a C/N ratio of 15 and C/P_2O_5 ratio of 10.

10. Nitrogen fixation occurs in the filter bed but results in the growth of film of low N content and low purification.

11. A low C/N ratio and a high concentration of organic matter promote the rapid development of the film and attainment of high purification. They also lead to excessive growth of the film and where possible should be used only in the first month of the working of the filter.

12. With a C/N ratio of 15 or less a filter bed 5 ft. deep will give complete purification of 0·2 % sugar solution at a rate of flow of 100 g.y.d. using coarse gravel or clinker. With a C/N ratio of 50 to 100 a much deeper filter would be required, and better results could more easily be secured by the addition of nitrogen than by deepening the filter.

13. Both stages of nitrification occur in the filter bed and are subject to considerable fluctuations not correlated with the supply of nitrogen in solution.

This subject is also being investigated from the biological standpoint by Mr D. Ward Cutler, Head of the Department, Miss L. M. Crump, and Miss A. Dixon, to whom the author wishes to express his great indebtedness for constant help in consultation and practice; also to Mr E. Hannaford Richards and the members of the Fermentation Department, and to Mr Warren of the Chemical Department, for the Kjeldahl determinations.

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for	Q_{O_2}	−217	−855	−1277	−1723	−5771
substitute	Q_{O_2}	−1·5	−5·9	−8·8	−11·9	−39·8

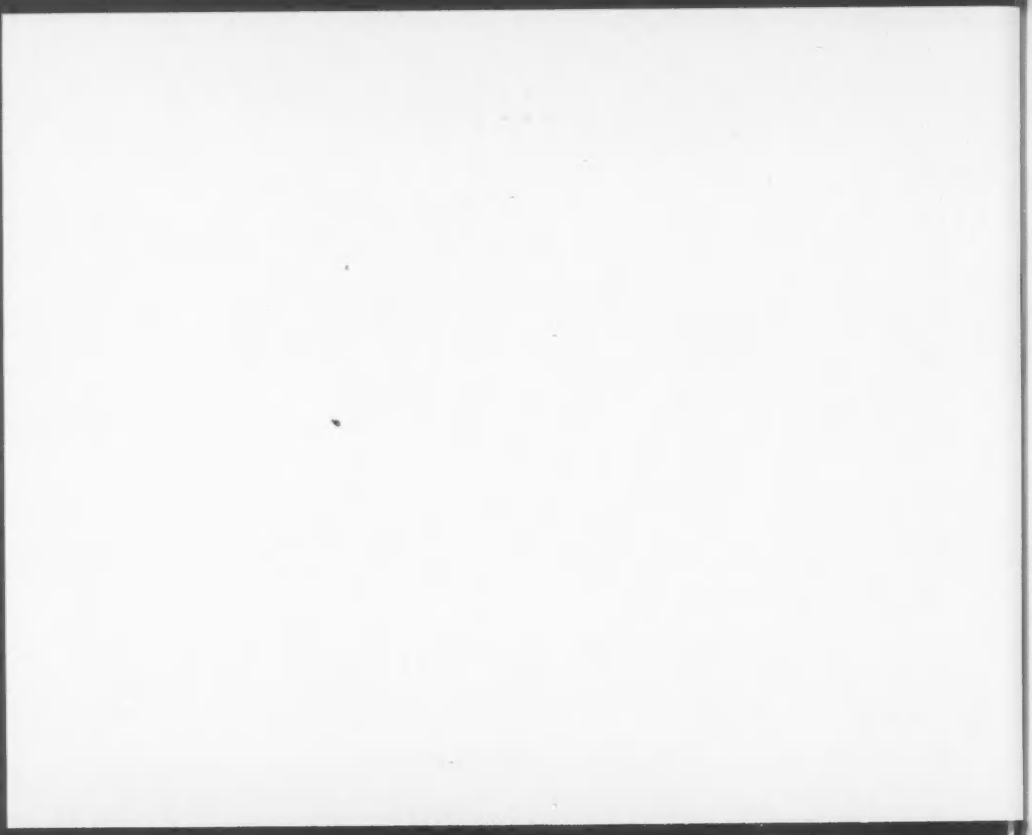
Line 3:

for dividing *substitute* multiplying

for the third and fourth sentences

substitute It will be seen that these values are of the same order as those found for yeast and mammalian tissues,

Page 887:

for part 2 of summary

substitute The rates of oxidation are of the same order as those found for yeast and mammalian tissues.